Establishing Novel Immunoaffinity Purification Methods for Fusion Proteins

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Abstract

The experiments in this thesis were performed to determine if novel uses of three fusion proteins could be established as a means of improving the protein purification process, and in particular, the elution step, thus resulting in the establishment of novel immunoaffinity purification methods.

There are numerous fusion tags currently available for use as purification tools. Many of these current methods for protein purification require harsh elution steps, such as a low pH elution, which can be harmful to the protein. There are few purification methods that successfully purify protein, while maintaining a gentle pH environment for the protein.

The goal was to employ a new in-house tag, and enhance two previously established tags, to optimize purification methods that would not require low pH elution steps. For each fusion tag in this work, there were three major aspects to the establishment of the purification method. The first aspect was the expression and purification by Nickel-IMAC of the fusion protein. The second aspect was the expression, purification, and antibody selection of an antibody specific for the fusion tag. The third aspect was the purification of the fusion protein on an immunoaffinity column to which the antibody was immobilized.

The first fusion tag was a peptide-tag derived from the C-terminus of Protein Y. It was to be used as a Flag™-tag replacement. An antibody was selected based on ELISA results and a Protein A-bound antibody small-scale purification test. The peptide-fusion protein was successfully purified in PBS, by competitive elution with excess synthetic

peptide, using the selected antibody in an immunoaffinity column format. Though column capacity was low, yet equivalent to an anti-Flag resin, it did result in pure protein.

The second fusion tag was GFP. An already established fusion tag, it is sensitive to low pH, therefore mutations were made to an anti-GFP antibody in order to convey pH-dependent binding. The antibody was chosen based on Octet Kinetic Analysis and Protein A-bound antibody small-scale purification tests. The GFP-fusion protein was successfully purified in non-extreme pH conditions using the selected antibody in an immunoaffinity column format. The capacity was better than that of the previous method, though the purity may have been slightly less.

The last fusion tag was a ZZ-tag, which is also a previously established tag. A synthetic version of the B-domain of Protein A, it was used for its ability to bind murine IgG1 in particular conditions, and its weaker affinity for murine IgG1 in PBS. After yielding difficult to interpret results from Octet Kinetic Analysis, a number of murine IgG1s were tested as possible immunoaffinity antibodies in an immunoaffinity format. The low capacity and recovery made it difficult to support this method for future use.

The successful fusion protein purification by two methods in neutral pH conditions offers two new tools for purification of difficult to purify and pH-sensitive proteins, as well as a new tool for better purification of lysates. In particular, these methods will be useful for Biogen, as the established immunoaffinity antibodies are available in-house, providing low-cost options, as well as less harsh options, for future protein purification projects.

Biographical Sketch

The author is a 2006 graduate of Boston College. She is a New Hampshire native who grew up participating in numerous outdoor activities, including hiking, fishing, and playing soccer. She hiked Mount Washington in 2001 and completed a thirty-mile three-day backpacking trip in the Colorado Rockies in 2014. She caught both a wild rainbow trout and a Kenai River salmon in Alaska in 1997. She played soccer for the majority of her life, including on the Trinity High School 2001 NH Girls' Class L State Championship team. She has also been a serious long-distance runner since high school, and recently trained for and completed her first marathon in January 2016.

After twelve years of living and working in the Boston area, she has returned to live, work, and play in the state from which she originally hails. Her rescue dog, Kepler, joins her in enjoying all that New Hampshire has to offer. She looks forward to using her upcoming free time to further enjoy activities, both new and old, including cooking, reading, painting, running, gardening, and spending time with her family and friends, of both the two-legged and four-legged variety.

Dedication

I would like to dedicate this work to my parents, Gerald and Linda Kirouac, and to my grandparents, Camille and Claire Kirouac, and Daniel and Phyllis Webb. Without you all I would not be who I am today, nor would I be where I am today. Thank you for everything you have ever done for me. There are no words to express how much I appreciate the sacrifices you have made for me, the belief you have had in me, the support you have given me, and the love you have shown for me. I owe you everything and I love you all so much.

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Introduction

1.1. Research Problem

In the pharmaceutical and biomedical fields, the end goal is nearly always a proven, safe and efficacious treatment. The treatment may range from merely easing or curing a symptom, such as a mild itch, or may be much more advanced and life changing, such as a cancer therapy that acts as a tumor blocker to inhibit proliferation of cancer cells. In either case, years of research go into the final product, and throughout the course of such research, purified recombinant proteins are required for each step along the way. Purified recombinant proteins are an invaluable resource across the biomedical field, and in many cases, especially more recently, the protein itself is the end target of many clinical drug companies. Although most current clinical drugs are classified as small molecules, there are quite a few, upwards of 10% and growing, that are classified as biologics, and these are virtually all characterized as proteins (http://www.bayerpharma.com/en/research-and-development/technologies/small-and-large-molecules/index.php, 2015).

Purification of recombinant proteins is therefore an important aspect of biological research. Ease of purification is not to be taken for granted. Other important aspects of recombinant protein expression to consider are cellular expression levels, solubility, and the planned usage of the particular protein. One way to achieve the best combination of expression level, "purifiability", and solubility of a protein is to add a tag. Tags are routinely fused to recombinant proteins and can be used to aid in purification, to promote

expression or solubility, or as a means of detection in various assays. Popular peptide tags include FLAG™ and poly-histidine (His); while oft-used protein tags include Glutathione S-Transferase (GST), Green Fluorescent Protein (GFP), and maltose binding protein (MBP) (Waugh, 2005). Several factors must be considered when selecting a tag for recombinant protein, including the protein's end use and its particular physiochemical properties. That being said, there are pros and cons to all tags. It is often a hit-or-miss process to determine the best tag for a particular protein.

In some cases, the tag may negatively affect expression levels (Terpe, 2002). Or it may alter the cellular behavior or activity levels of the protein (Wu & Filutowicz, 1999; Bauman & Church, 1999). Occasionally, tags can make recombinant proteins more difficult to purify, or modifications can occur that cause loss of recognition and low yield (Schmidt et al., 2012). Large tags, such as protein tags, can be metabolically burdensome on the expression system, and many can be difficult to remove following purification (Waugh, 2005).

Removal of the tag is an important aspect of fusion tags, particularly when the native form of the protein is needed in applications such as crystallography, animal studies, etc. To achieve this oft-necessary step a site-specific sequence may be included between the tag and the protein of interest. This sequence could be recognized by a variety of proteases, thereby allowing the tag to be cleaved from the protein following purification, by addition of the proper protease. Proteases useful for this step include PreScission, thrombin, factor X_a, TEV, or enterokinase (Terpe, 2002).

The difficulty that often arises in purifying recombinant proteins, both with and without tags, suggests that additional, novel, efficacious tag methods would be valuable for improving various aspects of the protein production process, from cellular expression

to protein solubility to ease of purification. The first tag that will be explored in this thesis is a novel peptide tag, which has not been published in the literature, and is based upon the N-terminal and C-terminal peptide sequences of a protein denoted here as "Protein Y". This peptide-tag is being pursued as an alternative to Flag™-tag purification, and makes use of proprietary antibodies that are available in large supply. Additionally, there are two particular affinity tags within the literature that have not been well explored for use in protein purification. One of these is the green fluorescent protein tag, or GFP, a green-emitting protein of jellyfish origin (Shimomura, Johnson & Saiga, 1962). Although GFP is routinely used for immunoprecipitation studies, there are limitations for use in preparative purification. The second tag is the ZZ-tag, which is a synthetic subunit of the Protein A molecule, with the ability to bind the Fc region of antibodies (Nilsson et al., 1987).

1.1.1. Proposed Solution

I propose a novel use of each of these, in order to improve the protein purification process, specifically the elution step, which is often the most difficult purification step. In particular, would like to fuse a small peptide sequence tag, derived from either the N- or C-terminal sequence of Protein Y, onto a recombinant protein, and determine if the peptide-tagged protein can be purified on an affinity column, to which has been immobilized an in-house antibody specific for the peptide sequence. The tagged-protein will be competitively eluted using excess peptide, allowing for a gentle pH elution. I would also like to assess if mutant forms of the camelid antibody cAbGFP4, the wild type version of which has strong affinity for GFP, could be used for immunoaffinity purification of GFP-tagged proteins with a neutral pH elution. Lastly, I want to determine

if ZZ-tagged recombinant protein can be purified on a murine IgG1-immobilized column with a gentle elution at a neutral pH, by making use of the tight binding that occurs between Protein A and murine IgG1 in high salt (NaCl), high pH conditions, but that is lost in low-salt (NaCl), neutral and low pH conditions.

Thus, the goal of this project is to establish novel uses of existing tags as a means to affinity purify recombinant proteins using gentle elution conditions at neutral pH. If any of these tags works as predicted, it will offer an additional purification method to protein chemists. These approaches are particularly attractive within the company, Biogen, as they make use of existing internal reagents, which are renewable. The successful adaptation of any or all of the proposed novel tag uses will offer alternative approaches to purification of difficult to express or purify proteins, particularly pH-sensitive proteins, and could offer simpler methods for a number of proteins in general.

1.2. Difficulties Related to Recombinant Protein Purification

Within pharmaceutical and biomedical companies, research is focused on developing a treatment for disease. The treatment may be one that alleviates symptoms of disease, or it may aim to inhibit the pathways of the disease, while yet others seek to cure people of a disease entirely. During a product's research phase, which lasts for many years, purified recombinant proteins are required. Recombinant proteins are used for a variety of reasons, including, but not limited to, screening targets, proof-of-concept studies, assay targets to prove or disprove binding or affinity, for elucidation of structure, for selection of a protein of interest, and as use as clinical drug targets for testing suggested drug therapies (Terpe, 2002).

Unfortunately, as important as pure recombinant proteins are to a project,

purifying them is not always a straightforward task. There are a variety of methods used to obtain purified protein of high quality. Just as all diseases are unique and unable to be treated in the same manner, so are all proteins. Often, a general method can be imposed on similar proteins. For example, many antibodies can be affinity purified on a resin column of Protein A, which recognizes and binds the Fc-region of antibodies (Forsgren & Sjoquist, 1966). However, this is not an all-inclusive purification method.

Further complicating the purification process are protein alterations. These alterations include post-translational modifications such as glycosylation or phosphorylation, as well as amino acid mutations within the protein sequence, and truncations of the protein itself. These changes are often deliberately generated to help determine an optimal protein design for an intended purpose. Frequently, the changes induce consequences to the protein's characteristics, including its structure and folding, its isoelectric point, its solubility, its hydrophobicity, its internal bonds, and its stability. The intentional or unintentional alteration can affect the protein's ability to bind to other molecules, as well as what methods can be used to purify it, and how successful such a method will be. In some cases, certain modifications cannot be used because they render the protein insoluble or otherwise unstable, or perhaps cause protein aggregation, all of which are poor qualities for drug therapies. In other cases, various purification methods must be attempted to improve purification ease, as well recoverability. Overall, purification of recombinant altered proteins can be very difficult and inefficient.

There are currently myriad acceptable methods and tools in standard use for recombinant protein purification. Most are chromatography-based methods that make use of a variety of protein characteristics in various manners, and utilize an assortment of tools, from affinity resins, to ion exchange columns, to hydrophobic interaction

chromatography, to size exclusion columns. Fusion tags are routinely added to proteins to aid in affinity purification. Creating a fusion protein, by fusing a tag sequence to the protein sequence, has a number of possible benefits. It can enable the purification of a protein that was impossible or difficult to purify without the tag. Tags can be useful in biochemical assays, by providing a means to track or label the protein. Lastly, some tags can improve the expression levels or solubility of proteins, allowing for increased recovery of purer protein. (Terpe, 2002; Waugh, 2005). On the other hand, tags are not perfect and all have drawbacks.

1.3. Current Recombinant Protein Purification Methods and Tools

Affinity chromatography, as its name implies, makes use of a specific biological affinity interaction that occurs between two molecules. Usually the interaction is reversible, and often occurs between a mobile group and an immobilized (typically on resin) group. Following the interaction in which the mobile molecule is captured on an immobilized column (by affinity), the complex can be washed to rid the sample of interest of impurities, and then the molecule of interest is eluted using a buffer that breaks the interaction between it and the immobilized molecule (Waugh, 2005).

In addition to affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography (HIC), and size exclusion chromatography (SEC) are also various methods of purifying protein, with different chemical principles behind them. While there are a handful of different chromatographic methods, as just mentioned, the main focus now will be on affinity chromatography tools. Since affinity chromatography is based on the interaction between two molecules, there are seemingly countless ways in which it can be applied. In the following section, a variety of affinity

tools, including resin and fusion tags, will be discussed, including what they are and how they work, as well as some of their pros and cons. Although, as previously mentioned, numerous peptide and protein tags currently exist, the focus of the sections following will be on Protein A, GFP, His, and Flag™Tag, as these will all be important aspects of the research design found in this proposal.

1.3.1. Protein A

Used in affinity chromatography methods, Protein A is a well-known protein that is now widely used for purification of Fc-bearing complexes, such as monoclonal antibodies and recombinant Fc-fusion proteins. Originally isolated by Jensen from *Staphylococcus aureus* extracts in 1958, further studies found it was a protein capable of binding immunoglobulins, and it was designated Protein A (Grov, Myklestad, & Oeding, 1964).

Forsgren and Sjoquist further studied the protein to determine exactly how Protein A reacted with immunoglobulin. They discovered that the reaction that occurred between Protein A and human globulin was not a true antigen-antibody immune reaction, since it occurred more strongly between Fc fragments and Protein A than between Fab fragments and Protein A (Forsgren & Sjoquist, 1966). The structure of Protein A was debated for some time; whether it may be tetrameric or otherwise, or consist of homologous or heterogeneous regions. More work was published in 1986 that solved the structure of Protein A. This work clarified the IgG-binding properties of the questionable fifth region (E) and suggested that Protein A was both structurally and functionally pentavalent in IgG-binding, containing five homologous regions, all with an affinity for human immunoglobulin. All five of the Protein A fragments bound to affinity columns

containing human immunoglobulin (hIgG) (Moks et al., 1986).

Protein A-immobilized resin is now a common tool used for affinity purification of immunoglobulins. The high avidity for Fc-regions, resulting from Protein A's five homologous IgG-binding domains provides tight binding and specificity. This specificity and high affinity make it possible to achieve high purity of immunoglobulins, even from very crude samples, such as those containing serum. It is also a great tool because antibodies can be purified without the addition or fusion of other tags. Additionally, Fc-fusion proteins are often produced, such as extracellular domains of membrane receptors, and due to the affinity between Protein A and Fc, these fusion proteins gain the ability to be Protein A purified.

However, there are disadvantages to Protein A. Preparation of the protein can be both complicated and expensive. Therefore, to purify large amounts of protein by Protein A could be very costly. In addition, Protein A has varying affinities for different immunoglobulin species. For human species, it binds strongly to almost all IgGs, but not well to IgM. For murine species, it binds weakly to IgG1, but well to IgG2a, IgG2b, and IgG3. Despite its poor affinity for murine IgG1, Ey, Prowse, & Jenkin discovered that Protein A bound murine IgG1 at alkaline pH levels (Ey, Prowse, & Jenkin, 1978). In addition, Malm found high concentrations of salt (NaCl) also helped murine IgG1 bind to Protein A (Malm, 1987). In combination, it is now standard practice to bind murine IgG1 to Protein A using a high salt (3M NaCl), alkaline pH (around pH9) buffer, and elute it with a low salt, and low pH buffer (it usually elutes between pH 6-7); thereby making Protein A an effective murine IgG1 purification method after all. Although Protein A is able to bind a number of immunoglobulin species, it becomes necessary to elute the protein of interest with a low-pH buffer, which can be too harsh for pH-sensitive proteins,

and can occasionally lead to precipitation issues or cause loss of activity.

1.3.2. GFP

Green fluorescent protein, or GFP, is a bioluminescent protein of 26.9-kDa that was discovered in the jellyfish *Aequorea victoria* in 1962 by Shimomura, Johnson and Saiga (Shimomura et al., 1962). This protein emits a visible fluorophore that appears green, a quality that is extremely useful in the scientific world. Since its discovery and subsequent studies that revealed information about its structure, size, and function, it has been found to have a number of useful functions in the scientific community. Because it emits a visible green fluorophore, its presence can be visibly detected. Therefore, any proteins to which it is fused become detectable. This is useful for visibly determining protein and gene expression levels, as well protein localization detection, and for detection in various assays (Tsien, 1998). Additionally, based on its loss of absorbance and shift in excitation at extreme pH levels, it can be used as a physiological indicator (Patterson, Knobel, Sharif, Kain, & Piston, 1997).

Beyond its uses in tracking protein, GFP can be fused to proteins to act as a fusion tag. GFP-fusions can be bound to columns to which anti-GFP antibodies are immobilized. Unfortunately, the anti-GFPs in existence have very high affinity for GFP, rendering GFP nearly useless for preparative protein purification, as the extremely high affinity between GFP and anti-GFP make it almost impossible to remove GFP-fusions from the anti-GFP IP affinity column. This necessitates harsh elution conditions, such as eluting with reducing buffers at low pH, to separate protein from resin (Rothbauer et al., 2008). Not only is that potentially damaging to protein, but GFP is also known to be pH-sensitive, and higher pH levels can change the absorbance and excitation amplitudes of

the protein, while low pH levels can diminish the fluorescence, and cause loss of stability, leading to precipitation (Patterson et al, 1997; Tsien, 1998). Because of these limitations, the GFP-tag is not routinely used for preparative purification, but rather in applications where elution is not required, such as for small-scale study of protein-protein interactions where elution from IP resin is not necessary. This makes a GFP-tag difficult or impossible to use in applications when pure protein is required.

However, in addition to the GFP antibodies that have been used for years for these crude protein preparations, there has recently been development of a new kind of antibody, specific for GFP. This new antibody is a nanobody, a very small antibody 15kD in size. Because of its small size, it can be produced in a number of expression systems. It is also very stable. Nanobodies are derived from camelids. The camelid species are members of the biological family Camelidae, including camels, llamas, and alpacas; and they have unusual antibodies. Their IgG antibodies have no light chains, and only one antigen-binding domain on their heavy chain, and thus are known as heavy chain antibodies, or hcAb. In addition, the Fab region of these antibodies, to which antigens are bound, consists of only one domain, the variable domain of heavy chain antibodies, or V_HH, also known as a nanobody. It is the smallest known functional antigen-binding fragment (Saerens et al., 2005; Rothbauer et al., 2008; http://www.chromotek.com/about-us/the-alpaca-antibody-advantage/, 2015).

Early work published on camel single-domain antibodies demonstrated that V_HH structures could be altered to express V_HH antibodies specific for a variety of antigens, including GFP. In this work, the V_HH specific for GFP was termed cAbGFP4, and its sequence was published. The affinity between GFP and cAbGFP4 was also studied, and found to be fairly tight, at 0.32nM (Saerens et al., 2005).

Thus, GFP-fusions are useful in producing highly pure protein samples, based on the tight binding and degree of specificity to which anti-GFP binds GFP. However, this tight binding and specificity also results in protein that cannot be separated from the resin, so while pure, it cannot be used for much more than experiments to establish expression, because the beads are often included in the final sample.

1.3.3. His-tag

In 1988, E. Hochuli and his team at Roche, discovered a method for purification that combined the molecular characteristics of the amino acid histidine, with a recombinant protein of interest, in the hopes that the chemical characteristics of histidine could be exploited for purification benefits. Hochuli et al., found that adding a poly-His tag to a recombinant protein enabled the protein to be purified using the metal nickel's affinity to selectively bind adjacent histidine residues. When charging a metal chelate adsorbent with nickel ions, the histidine-tagged protein of interest bound to the adsorbent. The metal-charged adsorbent (in column format) was then washed with a neutral-pH buffer, allowing contaminating, untagged proteins, to flow through, while the protein of interest remained bound. Following that step, the column was eluted with a lower pH buffer that knocked off the protein of interest from the nickel-charged adsorbent (Hochuli, Bannwarth, Dobeli, Gentz & Stuber, 1988).

Hochuli et al. experimented with the number of histidine residues to include in a tag, from two to 6, as well as tag location, at the N- or C-terminus of the recombinant protein of interest. They also experimented with which buffers were best for the most efficient elution of protein. Overall, they found the combination that resulted in the best affinity rate and best recovery rate was when either a bi-His tag was attached to the

protein of interest's C-terminus and eluted with phosphate buffer between pH5.5 and pH6.5; or when a hexa-His tag was attached to either end of the protein of interest (N- or C-terminus) and eluted with guanidine hydrochloride (GuHCl) at pH5.0 (Hochuli et al., 1988). Therefore, using their method, one must take into account where the termini are located in the native structure of the recombinant protein of interest, and whether or not the protein needs to remain in a physiological buffer such as phosphate during elution. Although Hochuli and his team performed IMAC elution on His-tagged proteins using buffers at a pH between 5 and 6.5, the more common method of elution nowadays is by competitive elution using high levels of imidazole, in which the imidazole will compete with the His for binding to the metal resin, allowing the His-tagged protein to elute from the affinity column (Porath, 1992).

There are a number of benefits to the His-tag, some obvious, others less so. As seen in the work of Hochuli et al., the protein can be eluted in relatively mild conditions, including using physiological buffers, as well as at a more neutral than acidic pH (Hochuli et al., 1988). For another thing, the existence of multiple sequential histidine residues is a phenomenon rarely found in wild type proteins, so the addition of a poly-His tag to a recombinant protein for purification or separation purposes is relatively specific. Similarly, it is also a fairly efficient purification method. In addition, this tag is considered a peptide tag, as it consists of a small number of identical amino acid residues. Because the properties of affinity rely only on the existence of histidine residues, and not on any formational or structural characteristics, the purification via nickel immobilized metal affinity chromatography (IMAC) can be performed under reducing conditions, reducing the protein as far down as its primary structure, or amino acid sequence. This is not true of many other purification methods, such as immunoprecipitation with

antibodies. The cost of resins when using the IMAC method is also inexpensive compared to other purification methods. Due to its small size, it typically has minimal, if any, effect on the physical or chemical characteristics of the recombinant protein itself. Lastly, the His-tag has been found compatible with a variety of expression systems (prokaryotic, eukaryotic; mammalian, insect cell, e. coli), making it a universally available tag (Terpe, 2002).

Although there are numerous advantages to using the His-tag for protein purification, there are disadvantages as well. Because the His-tag relies on IMAC, a metal ion-charged column can result in metal leaching during purification, contaminating the protein. As nickel is potentially carcinogenic in humans (http://www.epa.gov/airtoxics/hlthef/nickel.html, 2013), it is particularly important to ensure that no leached nickel remains in the final product when pharmaceutical-grade protein products are involved. Or IMAC should be avoided altogether as the purification method in cases such as these. Histidine is also one of a number of amino acids that is vulnerable to oxidative reactions. The result of oxidative reactions is the production of highly reactive radical intermediates that can be damaging to a number of biomolecules, including proteins and amino acid side chains. The damage to proteins and amino acid side groups that occurs due to these oxidative reactions can cause loss of activity, aggregation, or other negative protein interaction effects (Krishnamurthy, Madurawe, Bush, & Lumpkin, 1995). Lastly, despite the fact that histidine tags are so small and have little, if any, effect on the functionality or structure of the protein to which it is attached, it is not guaranteed to have no effect on its protein, as found in a few cases. Goel et al. found that a C-terminal addition of a His-tag reduced activity (in this case, affinity towards the antigen) of an engineered single-chain Fv construct to as little as 30% of its

non-tagged counterpart (Goel et al., 2000). While studying the *pir* gene of plasmid R6K, Wu and Filutowicz also found largely different results between the his-tagged and non-tagged version of a protein. They found that the addition of a his-tag to a mutated version of protein caused dimerization of the protein, which negatively affected its binding ability. This indicates that the addition of a mere His-tag can instigate a huge conformational change, leading to remarkably different cellular behavior (Wu & Filutowicz, 1999). In yet another example, the C-terminal addition of a His-tag to Heparin Cofactor II more than doubled the thrombin inhibitory ability of that heparin II. In the same study, more experimentation was done that showed that the same tag on Heparin Cofactor III had the opposite effect, and actually reduced its thrombin inhibitory ability (Bauman & Church, 1999). Another limitation to the His-tag is the limit to the degree of purity it is able to achieve. When using IMAC columns, such as Nickel, Zinc, or Cobalt, it is possible for other proteins, not just the recombinant protein of interest, to bind to the resin, most often because of a high level of naturally occurring histidine residues found in proteins (Saraswat et al., 2013).

Despite these cautionary examples, it is true that in the majority of instances, the His-tag has no negative effects. However, it is still often necessary to remove the tag from the recombinant protein following purification. In many unaffected cases, the tag can be left attached, but for the production of pharmaceutical-grade protein, the tag must be removed. This additional step can be achieved by including a cleavage sequence between the tag and the protein that is specifically protease-recognized. This has possible negative consequences if the sequence is found elsewhere in the protein, or if the sequence is flanked by particular amino acids, or if it is found in a particularly difficult location within the folded protein, or if the protease cuts the sequence at a point that will

leave behind non-native amino acids (Terpe, 2002).

1.3.4. Flag™ Tag

In 1988, a group at the University of Washington, led by Thomas P. Hopp, worked to find a method for protein detection and purification that would not yield the usual undesired consequences that affected fusion proteins up to that point. Such unintended consequences they aimed to solve included the protein misfolding that led to inactive proteins, caused by harsh denaturants used in the elution process; the inability to remove the added sequence from the protein of interest without the use of harsh chemical conditions; and the difficulty found in eluting proteins by immunoaffinity purification with a specific antibody, due to the tight binding of antibody to epitope. Their discovery of the Flag peptide was based on a variety of factors (Hopp et al., 1988). The Flag peptide consists of eight amino acids: aspartic acid (D), tyrosine (Y), lysine (K), four aspartic acids (DDDD), and lysine (K), or "DYKDDDDK". Hopp et al. chose to limit their polypeptide to eight residues because eight amino acids can be encoded by a single oligonucleotide, and trypsinogen prosequences are all no longer than eight residues. Their goal was to use the enzyme enterokinase to later separate the peptide tag from the protein, based on the enterokinase cleavage site incorporated into the tag. Enterokinase is a trypsin-activating enzyme, hence the trypsinogen prosequence model of eight residues. The enterokinase specificity site is "DDDDK". The three amino acids "DYK" inserted between the protein of interest and the 5-residue enterokinase specificity site would hopefully act as a buffer to separate the protein and minimize any effects. They chose the 3rd residue of the "buffer" sequence prior to the specificity site to be K, based on the fact that the addition would yield the 6-residue sequence "KDDDDK" (Hopp et al., 1988). In

1981, Hopp and Woods had created a scale that ranked the hydrophilicity of all 20 amino acids. They did this because of their knowledge that charged hydrophilic amino acid side chains are common features of antigenic determinants, or the antigen's epitope. According to the values derived from their experiments, lysine (K) and aspartic acid (D), each had the highest recorded hydrophilicity value of 3.0, therefore this 6-residue sequence has a maximum value of hydrophilicity (Hopp & Woods, 1981).

A highly hydrophilic nature is a positive quality of a possible tag. In the protein's folded form, the hydrophilic marker will always be exposed on the surface of the protein, allowing for easy detection later. A surface-exposed tag also suggests less protein misfolding. Additionally, the aromatic amino acid tyrosine, Y, (included prior to KDDDDK), is also known to facilitate the interaction between antibodies and antigens, another useful property for later purification steps. Lastly, the first aspartic acid (D) of the peptide sequence, prior to the tyrosine, would further complement the antibody-antigen affinity property brought on by tyrosine, as tyrosines surrounded by charged residues (such as aspartic acid) were found to be highly involved in antigenic sites (Hopp & Woods, 1981).

After they determined the sequence they would use for their tag, Hopp et al. engineered the 8-residue peptide marker N-terminally on the lymphokine interleukin 2 (IL-2) to enable them to produce a monoclonal antibody, dubbed 4E11 – and now known as M1 – that was specific for their tag sequence DYKDDDDK. They performed experiments to show that mAb M1 could bind the fusion protein containing the peptide marker, and the protein could be eluted mildly from M1 with the removal of calcium from solution. In addition, they found that subsequent to M1 purification steps, they were able to remove the marker from the protein of interest, by treating with enterokinase, with

little if any negative effect to the protein itself, both physically and functionally (Hopp et al., 1988).

The discovery of this peptide marker, which was trademarked Flag™, was a beneficial tag for protein purification systems. Its small size had the benefit, like the His tag, of bringing relatively little interference to the protein itself, in terms of both form and function. Its ability to be mildly eluted during the purification process is useful for less stable proteins, and for any protein upon which harsh conditions are harmful. The relative ease with which the tag is able to be removed is also extremely beneficial, as there are many instances in which proteins are necessary in native form only, without addition of any non-native amino acids, such as this peptide tag. The Flag™ can be fused to the protein on either the N- or C-terminus, a factor adding to its flexibility (Terpe, 2002). It is also possible that the addition of Flag™ to a protein of interest can improve expression levels, at least in yeast expression systems. This is a benefit for difficult to express proteins, or proteins of which a large purified quantity is required. In addition, Flag™ can be used in a variety of expression systems, not only in *E.coli* and yeast, but in CHO cells as well. In addition to the 4E11 antibody, which was produced alongside original production of the Flag™ by Hopp et al. in 1988, there are a number of antibodies that have been created to recognize the marker (Einhauer & Jungbauer, 2001). Not only is this important for allowing a variety of antibody-specific purification methods, it is also useful in that some of the antibodies are position-dependent, and will only recognize the marker when it is N-terminally fused.

As noted above, there are some extreme benefits of the Flag tag, but there are drawbacks as well. For one thing, Flag™ is more advantageous when fused to the N-terminus of the protein than to the C-terminus, however the anti-Flag antibodies that

recognize Flag™ only N-terminally, such as M1, are of inadequate use when the N-terminal Flag™ is preceded by a methionine residue, or when it is C-terminally located (Einhauer & Jungbauer, 2001). This decreases the options of antibodies in such cases. Additionally, although Hopp et al. found that elution of the tagged protein from anti-Flag mAb M1 was possible when calcium was removed, this is not always the case, as some anti-Flag antibodies are not calcium dependent, such as anti-Flag M2. In such cases, the better elution option for fusion proteins was by competition with Flag peptide, or with a low pH solution (Einhauer & Jungbauer, 2001). Unfortunately, low pH can result in potentially harmful effects on the protein of interest, such as instability. Competitive elution by peptide is the more commonly used approach, using either the peptide itself to competitively bind to the antibody-column and knock off the peptide-fusion protein, or polymerized versions of the peptide. This approach will be investigated later, within the third aspect of the proposal.

The removal of Flag™ can be tricky as well. Hopp et al. found that the addition of the enzyme enterokinase would cleave Flag™ from the protein, because enterokinase recognizes the sequence DDDDK and cleaves at the C-terminal end of the K residue (Hopp et al., 1988). This is great when the Flag™ is N-terminally fused to the protein, allowing the entire tag to be removed with one enzyme. However, when the Flag™ is added C-terminally, enterokinase is useless in removing the tag. In order to remove a C-terminal tag completely, it may be necessary to insert a proteolytic cleavage site, which adds both another step to the purification, and additional amino acid residues to the protein itself.

Another disadvantage to the Flag™ is the fact that anti-Flag antibodies, despite their high specificity and affinity, when immobilized to resin, have a low binding

capacity for Flag™ (Waugh, 2005). This means that either a lot of anti-Flag is necessary for purification, which is expensive; or the purification needs to be done batch-wise, which is time-consuming; or the protein yield is low, which is inefficient.

In addition, Schmidt et al. showed recently, in 2012, that the addition of the Flag™-tag in insect cell expression systems is actually not beneficial at all. They found that when Flag™ was used in the insect cell expression system, a post-translational modification occurred, specifically the sulfation of the tyrosine of the sequence. This resulted in loss of recognition of Flag™ by the anti-Flag antibodies, to the extent that less than 20% of the protein was recovered in the purification step (Schmidt et al., 2012). The recognition of Flag™ by anti-Flag antibodies is one of the most important aspects of the Flag™ system, as that is how the tagged protein is recognized and purified following expression. Without the affinity between Flag™ and anti-Flag antibodies, protein purification with they system would be impossible. This indicates that Flag™ usage, at least in insect cells, is not advisable or prudent (Schmidt et al., 2012).

1.4. Could Novel Uses of Existing Fusion Tags Improve Affinity Purification Methods?

So far, the focus of discussion has been on a few affinity tools used in the purification process. The properties of such tools are exploited for purification purposes in a variety of ways. The tags are used for numerous reasons. Some allow proteins to be purified in a neutral manner without affecting the protein of interest or denaturing it. Others add an important quality to the expression of the protein of interest, such as increasing expression levels, or improving solubility. Others are small and therefore are minor perturbations that will have little affect on the protein when left attached and/or

will have little metabolic burden during expression. Some will require a particular purification method, known to be inexpensive and/or easy to scale up. Some can be added to the protein at either the N- or C-terminus. Some tags can be effective no matter what condition the protein is in, native or denatured. Others are highly specific, allowing a very pure protein to be purified. Some bestow high purification yields due to the nature of the tag. There are a number of benefits that different protein tags have in terms of purification and expression of protein.

There are also a number of disadvantages that come with the different tags. Some are large and therefore implicate a high metabolic burden on the expression system. Some are not very specific, and therefore a number of steps need to be performed to purify the protein, resulting in low protein yield, or a less-pure sample. Some necessitate expensive affinity resins for purification steps. Others require very harsh elution conditions, which could be harmful to the protein of interest. Some can only be added to a specific terminus of the protein. Whatever the tag chosen, for whatever advantage it yields to the protein, there is nearly always a disadvantage of some degree that comes along with it.

It is unlikely that there will ever be a tag that is considered perfect: one that adds to or improves upon a variety of qualities of the protein, without adding its own degree of disadvantage. The goal is therefore not to discover the perfect tag, but to add to the tag "toolbox" to increase the diversity of available tags, or to allow for more flexibility when it comes to tagging proteins. To find a reliable tagging system that combines a number of advantages would be a benefit to the scientific community. The focus of this work will be to establish novel methods of purifying ZZ-fusion, GFP-fusion, and novel-peptide-fusion proteins using gentle elution conditions.

1.4.1. Protein Y-Peptide Tag: An Alternative Peptide Tag to Flag™?

The Flag™ tag, although useful in a number of ways, has its drawbacks. One major drawback is the high expense of the anti-Flag resin. In general, it is quite useful due to its small size and specificity, allowing for easy fusion protein production and recognition by anti-Flag antibodies. For this body of work, I would like to create a Biogen version of the Flag™-tag. Its usefulness in immunoprecipitation is desirable, as well as its ability to be eluted without a low pH buffer. It would therefore be ideal to create a peptide tag similar to Flag™, in the sense that it is small and has antibodies specific for it. In addition, if the peptide-fusion can be competitively eluted off an antibody resin using a peptide elution instead of low pH, it will be suitable for our purposes. Since Biogen currently has in-house antibodies against a variety of peptide tags, these would be the ideal peptide sequences on which to focus. Having an in-house antibody provides an inexpensive alternative to the Flag™tag/M1 antibody combination, particularly because Biogen does not have access to a CHO clone expressing the M1 antibody, and therefore allows for scalable purification, because it would be easy to express and prepare the antibody and create antibody-coupled resin from it.

I propose that a previously unexplored peptide-fusion protein can be eluted by use of excess peptide, allowing for a gentle pH elution of the target protein. To meet this goal, a small peptide sequence tag, derived from either the N- or C-terminal sequences of "Protein Y", will be fused onto a recombinant protein with the goal of purifying the peptide-fusion protein on an immunoaffinity column, to which has been immobilized an in-house antibody specific for the peptide sequence.

There are two peptide sequences, both found within the full-length sequence of Protein Y, which have been targeted for antibody recognition. One sequence is found at

the C-terminus of Peptide Y, and the other is found at the N-terminus. Antibodies were generated against these as part of a drug discovery project to determine terminal antibodies specific for the protein of interest (Protein Y) as a means of better understanding the biology of this target. The antibodies were developed by injecting mice with the peptides of interest.

Either of these peptides tags will be used to create a fusion protein tag. By the same principle that the Flag™ tag is applied in purification steps, so will these peptide sequences. After creating successful fusion proteins, they can be purified using an antibody affinity column for the Peptide Y peptide sequence. The N-terminal or C-terminal peptide antibody (NT mAb or CT mAb) will be coupled to a sepharose resin to create an affinity column, to which the fusion proteins can be loaded and bound. Instead of eluting with low pH buffers, excess peptide can be loaded as a competitive elution step to knock the fusion protein off the antibody column. This will achieve two purification goals: a neutral pH elution, and the establishment of an inexpensive in-house method, since the monoclonal antibody against the peptide targets can be created in-house, with unlimited availability.

1.4.2. Establishing a cAbGFPmutant Affinity Resin and an "Eluteable" GFP-Fusion

Based on the knowledge that cAbGFP4 binds tightly and specifically to GFP, and the fact that the cAbGFP4 sequence is defined and published, the goal is to create a purification strategy using a mutant form of the cAbGFP that will allow for elution of GFP-fusions at more neutral conditions. Since the sequence is published, it enables us the ability to express large amounts of it. Because cAbGFP binds tightly to GFP, an amino acid mutation, or possibly two, somewhere in the GFP-binding site or in a site

surrounding the binding pocket, could affect binding strength between the two molecules. Mutations have indeed been identified by others (Arndt, Dalkilic-Liddle) that confer pH-dependent binding of cAbGFP to GFP-fusion proteins, however their use as a purification tool has yet to be explored. There are a number of programs at Biogen for which preparatively purified GFP-labeled protein would be advantageous.

In order to create mutants, a variety of possible mutation sites have been established by other scientists at Biogen for use in in-house tech development projects, based on the pIs at the binding site of cAbGFP (Arndt, Dalkilic-Liddle). Subsequently, these other team members performed molecular biology techniques to design the proteins with a variety of mutations, and then express them in CHO cells. The numerous cAbGFP mutants will be tested for ability to bind GFP and dissociate from GFP at neutral pH.

Prior to creating a GFP-fusion protein, the binding between the selected mutant cAbGFP and GFP itself will be confirmed by me using kinetics (using Octet instrument). This will also help determine the off-rate of GFP from mutant cAbGFP in various buffers and help to identify some ideal mutant candidates. This should help establish a binding and dissociation relationship between the two entities, and allow us to determine if it is a viable purification scheme. The evaluation of these mutants will also help serve as a control, to determine that we have chosen the appropriate mutant for the purposes described here. Upon identifying an appropriate candidate or two, I will then test them in my proposed purification method, with the knowledge that a candidate that binds and elutes GFP as intended in buffer format during Octet analysis may not behave the same in immobilized column format.

Following the expression, purification, and subsequent binding analysis of the mutant cAbGFP, the mutants can be evaluated for their utility on a solid matrix. To do so,

they can then be used for immunoprecipitation of the GFP-fused recombinant protein, by coupling mutant-cAbGFP to a sepharose resin to create an immunoaffinity column. The GFP-fusion protein will then be bound to the column at the optimal pH that allows for binding, and then eluted at the pH that has been determined to interrupt binding between the two molecules, based on the results of the binding studies. It is important to note, that although production of cAbGFP protein in high quantities would not necessarily be feasible in an academic lab, the nature of our company and our in-house capabilities allows us the tractability to express gram quantities of mutant-cAbGFP in CHO cells, easily and cheaply.

This approach, if successful, would be beneficial for the company and could benefit a number of projects that rely on quality protein that may be difficult to purify in more extreme pH conditions.

Like with the ZZ-tag plan, described next, if a mutant cAbGFP can be created that allows for GFP to both bind to mutant cAbGFP-coupled affinity resin in a pH-dependent manner and elute at a relatively mild pH, it will be beneficial tool for the affinity purification of GFP-tagged proteins.

1.4.3. Using the ZZ-tag as an Affinity Tag for mIgG1-immobilized Resin

After the Protein A structure was solved, and more knowledge about its IgG-binding domains was gathered, further research was performed in order to create a synthetic form of its IgG-binding domain. In 1987, Nilsson et al. designed a synthetic domain, designated domain Z. The Z domain is derived from the B domain of Protein A. They chose the B domain largely because of the determination that methionine residues, common to Protein A domains A, D, and E, were what made Protein A sensitive to

cyanogen bromide. Additionally, the asparagine-glycine sequence at residues 28-29 in all five of the domains make the protein sensitive to hydroxylamine treatment. Removing these problems allowed for chemical cleavages to occur when gene fusions were created from such domains. In addition, domain B consists of the consensus sequence for the IgG-binding domain. Nilsson et al. were able to eliminate domain B's hydroxylamine sensitivity by mutating the 28-29 asparagine-glycine to an asparagine-alanine without negatively affecting Fc-binding. They also added a non-palindromic restriction enzyme site to the 5' end of the fragment, so that the fragment could be polymerized, enabling them to create a Z, ZZ, Z(V), Z(X), etc. Importantly, they determined that domains B and Z bind to IgG with comparable affinity, and each can be eluted from an IgG affinity column at the same pH (Nilsson et al., 1987).

There are multiple benefits derived from the development of domain Z. It is much cheaper than Protein A, and it can be used to purify IgGs since it retains affinity for the Fc region (Nilsson et al., 1987). In addition, it has been found to help prevent the effects of proteolysis, thereby reducing degradation of target protein, as well as increasing recovery of protein (Mergulhao, Taipa, Cabral, & Monteiro, 2004). Since it can be polymerized, it can be altered to increase binding capacity, by adding additional Z domains.

There is plenty of published work that describes the many current uses of the engineered Z- and ZZ-domains. The ZZ-tag has been used to increase both solubility and expression levels of proteins, and has outperformed other tags, such as His and GST, in that respect (Hammarstrom, Hellgren, Van Den Berg, Berglund, & Hard, 2001). It has been used numerous times as the targeted tag in IgG affinity chromatography steps, and also to competitively elute single Z-tagged fusion proteins, since ZZ binds more strongly

than Z to Fc (Nilsson et al., 1994). Additional published works make use of the ZZ-domain as an inexpensive immobilized resin for purification of immunoglobulins (Chen, Huang, Jiang, Pan, & Hia, 2006).

One other important aspect, in terms of the work presented here, is offered by Ljungberg et al. They further analyzed the Z tag, along with various Z constructs, such as ZZ and other multiplicities, and EB (domains E and B), etc., and they discovered that smaller proteins, of particular importance ZZ and Z, had five to ten times weaker affinity for IgG than Protein A (Ljungberg et al., 1993).

The Z-domain is the established result of others' engineering efforts. It already has a number of useful applications, as mentioned above. Therefore I do not aim to engineer the ZZ-tag, but instead implement and attempt a novel purification approach using the previously established ZZ-fusion tag. Based on formerly discussed knowledge regarding Protein A and the Z domain, I propose a slightly different, not well-explored approach that takes the established knowledge one step further to develop a purification strategy for ZZ-tagged recombinant protein that allows for neutral pH elution. After it was established that high pH and high salt (NaCl) concentration each improve the binding between protein A and murine IgG1 antibodies (Ey et al., 1978; Malm, 1987), it became standard practice to increase the pH and NaCl concentration of supernatants containing murine IgG1 prior to affinity-purifying them on a Protein A column. Since the affinity between Protein A and the murine IgG1 is weak when pH below 9 is combined with low NaCl concentration, the murine IgG1 of interest can be eluted from the column by almost completely removing NaCl from the elution buffer and dropping the pH. The murine IgG1 will end up eluting relatively quickly and in a relatively neutral pH.

Therefore, we can take advantage of the ZZ tag, since it has weaker avidity for

IgG than pentavalent Protein A, as illustrated by Ljungberg et al., and since Protein A can be bound to murine IgG1 under specific NaCl and pH conditions and eluted at neutral pH and low NaCl conditions, as demonstrated by Malm and Prowse et al. This knowledge can be exploited to create a purification strategy in which a ZZ-fusion protein can be captured on a murine IgG1-immobilized column and gently eluted at a neutral pH.

Instead of using a Protein A or ZZ resin to bind and elute a murine IgG1 protein of interest, a murine IgG1 immobilized resin column could be created by coupling a murine IgG1 to sepharose resin. That column could then be used to bind the ZZ-tagged fusion protein, by way of the mIgG1-ZZ interaction. Since murine IgG1 binds to Protein A tightly in high NaCl, high pH conditions, but elutes at low NaCl, mild pH conditions, the ZZ-tagged protein could be eluted from the column in a neutral pH setting, possibly with a buffer that imitates physiological conditions, such as PBS. This would have great purpose for any pH-sensitive proteins, such as kinases, and would generally allow for milder, gentler elution of any protein to which the ZZ-tag was fused.

Chapter II

Materials and Methods

2.1. Protein Expression

Before any work could be performed, a number of proteins needed to be expressed by CHO cells so that they could subsequently be purified and analyzed.

2.1.1. Fusion Proteins

For each fusion protein (ZZ-fusion, ProteinY-NTpeptide-fusion, and ProteinY-CTpeptide-fusion IDL130; GFP-fusion and ProteinY-CTpeptide-fusion IDL517; and Z-fusion IDL519), plasmid DNA was produced and passed along by the in-house molecular biology group, and stable transfections were performed in CHO DG44 cells using the DNA. All transfection and cell culture work was performed in a Baker SterilGARD e3 Biosafety cabinet. For each transfection, 6e6 CHO DG44 cells were centrifuged at 450xg for 5minutes. The pellet was re-suspended in 20mL of Room Temp (RT) Gibco CHO-S-SFM II (without hypoxanthine and thymidine) media (ThermoFisher, 31033-020). 12µg of plasmid DNA was mixed with 600µl CHO-S-SFM II media and 30µl FectoPro DNA transfection reagent (Polyplus 115-375) by pipetting, and complex left at RT for 10 minutes. The DNA mixture was re-pipetted and added to the 20mL RT cell and media mixture, then immediately vortexed to mix. 3mL of complete mixture was added to each well of a Corning Costar 6-well flat-bottom cell culture plate (3516), then stored in a static incubator at 37°C, 5%CO_2, and 80% humidity. After four days, 2mL media was aspirated from each well and exchanged with 2mL in-house proprietary media

"CHOM39", containing 1x ThermoFisher Antibiotic/Antimycotic (ThermoFisher, 15240-112). four days later, media was exchanged as previously. Cells were counted and viability analyzed on Beckman Coulter ViCell Cell Viability Analyzer (731050). When viability began increasing, cells were scaled up to 30mL at 2e5 cells/mL in a 125mL 0.2µm vented non-baffled plastic shake flask (Corning, 431143). Every 3-4 days, cells were split to same density and volume increased, until reaching final volume of 1L in a 2L 0.2µm vented non-baffled plastic shake flask (ThermoFisher, 4115-2000). All flasks were incubated in Infors HT Multitron Cell shaking incubator at 37°C, 5%CO_2, 80% humidity, and 140rpm. 72hours after reaching 1L volume, cells were fed with 5% volume (50mL / 1L shaker flask) in-house proprietary feed, "CF17", and were shifted to 28°C 48 hours after feeding. 14 days after expansion into 1L volume, cells were harvested by centrifuging at 3000xg and filtering through Parker Domnick Hunter Demicap Filter Capsule Propor PES membrane 0.2µm filter at 100mL/minute. Recovered supernatant was spiked with 0.04% NaN3 and stored at 4°C until further use.

2.1.2. Antibodies

The three various purification methods required three different antibodies that would have unique targets. All were produced, expressed, and purified in-house.

2.1.2.1. mIgG1s and anti-proteinY-peptide mAbs. Stable transfection and protein expression were identical to that of the fusion proteins, except that two plasmids were used for antibodies: 6µg of heavy chain plasmid DNA and 6µg of light chain plasmid DNA.

2.1.2.2. anti-GFP cAbGFPmutant camelid antibodies. Plasmid DNA came from same in-house source as that for fusion proteins, but small-scale (5mL) transient (not stable)

transfections were performed for 36 cAbGFPmutants, for preliminary expression of antibody material to be used for screening. Following screening, eight medium-scale (200mL) transient transfections were performed for cAbGFPmutant antibody expression. For each 1L final volume transient transfection (all reagents used at 1/5 of given amount for 200mL transfections), 6e9 healthy CHO-S cells were sterilely centrifuged at 450xg for 5minutes, and resulting cell pellet was re-suspended in 10mL transfection media, an in-house proprietary "CHOM39-NIC" media, with 20ng Long-R3 IGF (Sigma Aldrich 91590C) and 5µg/mL Transferrin (Sigma Aldrich T3705-1G). It was then transferred to a final volume (1L at 6e6cell/mL) of the same media, in a sterile, non-baffled, 0.2µm vented 2L plastic shake flask (Fisher 4115-2000) and stored in Infors shaking incubator at 160rpm, 37°C, 5%CO_2, and 80% humidity. While cells shook, 1mg of plasmid DNA was added to 50mL RT OptiPro SFM (Invitrogen 12309-050) and vortexed. Then 2mL FectoPro DNA Transfection Reagent (Polyplus 115-375) was added to mixture, complex vortexed, and let sit at RT for 5minutes. DNA complex and 1% volume of ThermoFisher antibiotic/antimycotic (15240-112) was then added to 2L flask of cells and returned to same incubator. 24hours later, temperature was dropped to 28°C. Cells were monitored and counted on Beckman Coulter ViCell Cell Viability Analyzer (731050) starting day 8, and harvested when viability dropped below 60%, on day 11, by filtering through Parker Domnick Hunter Demicap Filter Capsule Propor PES membrane 0.45um filter at 100mL/minute, and then adding 0.04% NaN_3 to clarified supernatant and storing at 4°C until further use.

2.2. Protein Quantification

All protein titer experiments for antibody quantification, and antibody-binding

kinetics experiments for determining antibody dissociation rates (see *Octet Kinetic Analysis*), were performed on Pall LifeSciences ForteBio Octet RED96 instrument using Protein A (ProA, 18-5013), Protein G (ProG, 18-5084), anti-penta-His capture (HIS1K, 18-5122), anti-human Fc capture (AHC, 18-5064), anti-murine Fc capture (AMC, 18-5090), or anti-murine IgG quantitation (18-5024) sensors. All samples were prepared in "Octet Buffer," comprised of 1mg/mL BSA, 1xPBS, 0.02% Tween-20 and 0.01% Azide.

2.2.1. Octet Concentration Analysis ("Dip and Read") Assay

To determine titers, the appropriate sensor was equilibrated in Octet Buffer, dipped into 300µl supernatant containing protein of interest, and read for 2minutes while shaking at 2000rpm at 30°C. Resulting titer estimate was used for determining column sizes in purification steps.

2.3. Protein Purification

For their use in testing the proposed immunoaffinity purification methods, the antibodies that targeted the fusion tags needed to be purified, so that they could be properly screened to determine the best candidate, and so that purified antibody was available for covalent immobilization to resin. In addition, the fusion proteins had to be purified by a standard purification method, in this case by Nickel-IMAC purification, as a control to which the test methods would be compared against.

2.3.1. Antibody Purification

For their use in immunoaffinity purification methods, the antibodies that targeted the fusion-tags needed to be purified for screening and for their use as an affinity resin.

The antibodies included the anti-GFP cAbGFPmutants, the anti-ProteinY-peptide mAbs, and the murine IgG1s for the ZZ-fusion.

2.3.1.1. cAbGFPmutant Antibodies. All of the cAbGFPmutant antibodies were purified by Protein A. Each cAbGFPmutant antibody was purified on a GE 5mL HiTrap rProtein A FF Sepharose Column (17-5080-01) depyrogenated with 0.5M NaOH and equilibrated in PBS. The protein of interest was loaded on the column using Cole Parmer Masterflex 4S Digital Economy Drive Peristaltic Pump with 8-channel multichannel pump head (7535-08) at 5 mL/minute. Column was washed with five column volumes of PBS. Antibody elution was performed manually on an AKTA Explorer (GE 18140300) for protein peak visualization. Antibody was eluted from the column with low pH elution buffer (25mM $NaPO_4$, 100mM NaCl, pH2.8) until A280 protein peak dropped to baseline. The purified fractions were pooled accordingly, neutralized with $1/20^{th}$ volume 0.5M $NaPO_4$ pH8.6, 0.22μm filtered, quantified by UV/Vis, and characterized by gel and analytical SEC.

2.3.1.2. anti-ProteinY-peptide mAb Antibodies. The anti-proteinY-peptide mAbs were also purified on Protein A, following the same protocol as described above. However, those purifications were completed previously, in-house, at larger scale.

2.3.1.3. murine IgG1 antibodies. The murine IgG1 antibodies for ZZ-fusions were also purified on Protein A, but in high pH, high NaCl conditions. Steps were same as described above, but sample was diluted 1:2.5 in 5M NaCl, 150mM Tris, pH8.9 before loading; and equilibration and wash steps occurred in 3M NaCl, 50mM Tris, pH7.8. Like the anti-proteinY-peptide mAbs, the anti-ZZ antibodies were also purified previously in-house, at a larger scale.

2.3.2. Nickel-IMAC Purification of Fusion Proteins

All of the fusion proteins were purified by Ni-NTA chromatography for comparison to the purification methods being tested. A clarified supernatant sample of each was spiked with 250mM NaCl, 50mM Tris pH8.0, 5% Glycerol, and 10mM Imidazole, and purified on a GE 5mL HisTrap Excel Sepharose Column (17-3712-06) depyrogenated with 0.1M NaOH at 5 mL/minute and equilibrated in equilibration/wash buffer (250mM NaCl, 50mM Tris pH8.0, 5% Glycerol, 10mM Imidazole) at 5 mL/minute. The protein of interest was loaded on the column using Cole Parmer Masterflex 4S Digital Economy Drive Peristaltic Pump with 8-channel multichannel pump head (7535-08) at 5 mL/minute. Column was washed with five column volumes of equilibration/wash buffer. Antibody elution was performed manually on an AKTA Explorer (GE 18140300) for protein peak visualization. Antibody was eluted from the column with high-imidazole elution buffer (250mM NaCl, 50mM Tris pH8.0, 250mM Imidazole) until A280 protein peak dropped to baseline. The purified fractions were pooled accordingly, 0.22μm filtered, quantified by UV/Vis, and characterized by gel and analytical SEC.

2.4. Antibody Screening

A variety of screening methods were used for each of the three sets of antibodies that would be used in the various test immunoaffinity purification methods. ELISA and Octet were used for binding studies, and resin analysis, in which antibody was bound to Protein A to test elution capabilities of various antibodies, was performed as an initial screen for column-format purification.

2.4.1. ELISA (Enzyme-Linked ImmunoSorbent Assay)

A panel of anti-proteinY-peptide antibodies were screened for binding by ELISA. two x 96-well flat-bottom ELISA plates (Thermo Fisher Pierce 15041) were coated with 100µl of 10µg/mL (1µM) of either C-terminal or N-terminal peptide in pH8.9 PBS buffer and stored at 4°C overnight. Wells were washed with 350µl PBST (PBS with 0.05% tween-20), then blocked with 350µl blocking buffer (1%BSA in PBST) for 2 hours at RT. Wells were washed as previously. Antibody (anti-proteinY-NTpeptide or anti-proteinY-CTpeptide) was added in seven x 1/3 dilutions, plus one negative control containing no antibody (100µl/well, dilutions were made in 1%BSA/PBS) and left 2 hours at RT. Wells were washed as previously. 100ul of a 1:10,000 dilution goat anti-humanFc HRP conjugate detection antibody was added (ThermoFisher, goat anti-human IgG Fc-HRP, H10007) and left 1 hour at RT. Plates were washed as previously. 100µl TMB substrate (ThermoFisher, 34028) was added to all wells to develop. The reaction was quenched with 100µl/well H_2SO_4. The absorbance was measured at 450nm on a plate reader, and analyzed with SoftMax analysis software.

2.4.2. Octet Kinetic Analysis

Kinetics testing involved multiple steps, including equilibration, loading, baseline, association, and dissociation. All experiments were performed at 30°C with shaking at 1000rpm. When possible, data was fit and analyzed on ForteBio's data analysis software to determine dissociation rate (K_D).

2.4.2.1. cAbGFPmutants. For cAbGFPmutants antibody K_D screening, anti-human Fc sensors were used. cAbGFPmutant antibodies were loaded onto plate at 100ug/mL (200ul) in Octet Buffer for immobilization (loading) to biosensor tips for 300 seconds. A

60-second baseline steps (200ul) occurred in Octet Buffer. The association step exposed the immobilized cAbGFPmutant antibodies to 10µg/mL (200µl) eGFP LPETG-His (189355-70) in PBS (pH7.0), or PBS pH5.5, or 20mM BisTris Propane with 150mM NaCl, pH9.5, for 300 seconds. The dissociation step occurred in Octet Buffer for 300 seconds.

2.4.2.2 murine IgG1s. For murine IgG1 K_D screening, conditions were identical to those in ProteinY-peptide mAb screening, except the following: Anti-human Fc capture (AHC), ProA, and ProG tips were used. The load step exposed anti-murine Fc capture tips to various murine IgG1 and mIgG2a antibodies. The baseline step occurred in Octet Buffer or high salt buffer (3M NaCl, 50mM Tris pH8.9). The association step exposed various immobilized murine IgGs to Z- or ZZ-tagged fusion proteins at 25µg/mL.

2.4.3. Protein A-bound antibody resin analysis

In the case of anti-ProteinY-peptide mAbs, it was necessary to confirm the ELISA results and determine if the results would translate to an on-column purification format. To do this, the antibodies were each bound to Protein A resin, as a way of binding them to a column, and they were used in this way to test purify some fusion protein. In the case of the anti-GFP cAbGFPmutant antibodies, it was helpful in determining which of the eight candidates that had promising Octet Binding Analysis results would translate best to column format. These on-column analyses were performed because Octet results do not always translate equally to a linear column format.

2.4.3.1. Anti-ProteinY-peptide mAbs. To further confirm and test binding capability of the six peptide mAbs, 200µg of each mAb and 60µl of Protein A resin (equilibrated in PBS) were mixed at RT for 30 minutes while shaking, in a Corning Costar Spin-X Plastic

Centrifuge Tube 0.22µm Filter (Corning, CLS8163). Following a PBS wash, 200µg of fusion protein IDL130, containing both ProteinY-terminal peptides, was added to each of the six Protein A-bound mAb resins, and mixed at RT for 30minutes. After washing with PBS, the fusion protein was eluted from the bead mixture with 100µM ProteinY-CTpeptide. Samples of resin, after fusion protein binding and after elution steps, were analyzed by gel, along with flow-through of each step.

2.4.3.2. cAbGFPmutant Antibodies. To narrow down cAbGFPmutant antibody candidates to a final candidate, the eight cAbGFPmutants chosen from Octet Kinetic Analysis were analyzed for elution ability by testing on Protein A beads. For each mutant, 0.5mg was mixed for 30 minutes at RT with 100µl Protein A beads equilibrated in PBS pH7.0, in a Corning Costar Spin-X Plastic Centrifuge Tube 0.22µm Filter (Corning, CLS8163). The Protein A-cAbGFP mixture was washed with PBS to remove non-bound cAbGFPmutant, then equilibrated in PBS pH5.5 before adding 0.5mg GFP-fusion and mixed at RT for 2 hours. The flow through was collected, and then the GFP-fusion was eluted with three 100µl fractions of 20mM BisTris-Propane, 150mM NaCl, pH9.0, followed by two 100µl fractions at pH9.5. The three most promising constructs were re-tested with the same method, except that only 0.2mg of cAbGFPmutant was mixed with Protein A beads, and elution took place with only pH9.5 buffer (20mM BisTris-Propane, 150mM NaCl, pH9.5). Once more the best construct was re-tested a third time, using 0.2mg cAbGFP and only 0.2mg GFP-fusion.

2.5. Covalent Immobilization of Antibodies to NHS-Activated Agarose

For each column that was coupled, 1mL Pierce NHS-Activated Agarose Slurry (ThermoFisher, 26200) was washed with 10mL ice-cold 1mM HCl, then equilibrated

with 3mL coupling buffer (0.2M $NaHCO_3$, 0.5M NaCl, pH8.3). Resin was mixed with 10mg of ligand (the immunoaffinity antibody) at 4°C overnight. Resin was washed with 5mL coupling buffer to remove non-bound groups, then washed with 5mL 0.1M Tris-HCl, pH8.3 to block non-reacted groups. Resin was washed with 3mL low pH phosphate buffer (25mM NaPO4, 100mM NaCl, pH2.8), followed by 3mL of high pH binding buffer (0.1M Tris-HCl, pH8.5), and the two steps were repeated twice more. Column was stored in 1xPBS with 0.04% NaN_3 until use.

2.6. Immunoaffinity Purifications of Fusion Proteins

As the final experiments in the thesis work, each of the proposed immunoaffinity purification methods was tested on a column covalently immobilized with the selected antibody, and fusion protein was purified with the column on an AKTA, so that results could be recorded for later observation and analysis.

2.6.1. ProteinY-CTpeptide-fusion Protein

1.0mL of coupled chi rabbit hIgG1anti-proteinY-CTpeptide mAb B40-NHS resin was put into a column, which was connected to an AKTA explorer, and the run was recorded with UV measuring absorbance at 280nm and 495nm. The column was equilibrated at 0.5mL/min with PBS pH7.0. A 10mL clarified sample of ProteinY-CTpeptide-fusion protein IDL517 was loaded through sample loop onto equilibrated column, washed with PBS pH7.0 until baseline flattened, and eluted with excess proteinY-CTpeptide (at 9.2mg/mL) until peak tapered off. Column was eluted with low pH elution buffer (25mM $NaPO_4$, 100mM NaCl, pH2.8) to remove any non-eluted material. 500µl Fractions were collected in a Nunc 96-well deep well plate (Sigma

Aldrich, Z717266-60EA). Peak fractions were analyzed by gel and by UV/Vis (also see below), and the fractions were pooled appropriately and further analyzed.

2.6.2. GFP-fusion Protein

0.5mL of coupled anti-GFP cAbGFPmutant IDL652-NHS resin was put into a column, which was connected to an AKTA Explorer, and the run was recorded with UV measuring absorbance at 280nm and 495nm. The column was equilibrated at 0.5mL/minute with PBS pH5.39. 250µl 1M HCl was added to 10mL of GFP-fusion protein IDL517 clarified supernatant to drop the pH to 5.5. Sample was loaded through sample loop onto equilibrated column, washed with PBS pH5.39 until baseline flattened, and eluted with 20mM BisTris-Propane pH9.5, 150mM NaCl, until peak tapered off. Column was eluted with low pH elution buffer (25mM $NaPO_4$, 100mM NaCl, pH2.8) to remove any non-eluted material. 500µl Fractions were collected in a Nunc 96-well deep well plate (Sigma Aldrich, Z717266-60EA). Peak fractions were analyzed by gel and by UV/Vis (see *Quantification and Purification* below), and the fractions were pooled appropriately and further analyzed.

2.6.3. ZZ-fusion protein

Three different murine IgG1 antibodies, with affinity for Z and ZZ domains, were coupled to NHS agarose and tested in a gravity/drip column format, along with a mIgG2a antibody that served as a positive control for binding, but a negative control for elution. Each of the four columns were equilibrated with 5mL high pH, high salt buffer (3M NaCl, 90mM Tris pH8.9). The sample of either ZZ-fusion (IDL519) or Z-fusion (IDL130) was diluted 1:2.5 in 5M NaCl, 150mM Tris pH8.9 and loaded onto equilibrated

column. Columns were washed with 5mL high pH, high salt buffer and then washed with 3mL 3M NaCl, 50mM Tris pH7.8. The fusion-proteins were eluted with PBS pH7.0, and followed with low pH elution buffer (25mM $NaPO_4$, 100mM NaCl, pH2.8) to remove any non-eluted material.

2.7. Quantification and Characterization

To best compare the test purification methods to a standard purification method, all of the purified proteins were characterized in a variety of manners, including UV/Vis concentration, Gel-CHIP analysis or SDS-Page, and analytical SEC.

2.7.1. UV/Vis Concentration Determination (Concentration of Purified Protein)

Purified samples were quantified by UV/Vis on spectrophotometer Nova Biostorage Trinean Xpose™ Touch & Go Reader. 2µl samples were pipetted onto microfluidic Xpose slide, and analyzed. All protein samples were quantified at 280nm, and concentration was determined using the calculated extinction coefficients for each protein.

Beer's Law states that ($A = \varepsilon * b * C$), where A = Absorbance, ε = wavelength-dependent molar absorptivity coefficient, b = path length in centimeters, and C = analyte concentration (mol/L or molarity). It was extrapolated to $C_{molarity} = A / (\varepsilon * b)$. Also, $(\varepsilon_{molar}) * 10 = (\varepsilon 1\%) x (MW \text{ of protein})$. Therefore $\varepsilon 1\% = (\varepsilon_{molar} * 10)/(MW)$. To convert concentration to mg/mL, $(A/ \varepsilon 1\%) * 10$ = concentration in mg/mL. For our purposes, the extinction coefficient (ε*b) was pre-calculated, so to calculate $C_{mg/mL}$, $C_{mg/mL} = A/(\varepsilon * b) = A_{280}/(\text{extinction coefficient})$. For example, the A_{280} for Nickel-purified IDL517 was 1.49, and extinction coefficient is 0.85. Therefore, $C_{mg/mL}(IDL517) = 1.49/0.85 = 1.75 mg/mL$.

2.7.2. Gel-CHIP analysis/SDS-Page

Selected purification fractions, and all final purification pools, were analyzed for purity by gel in one of two ways: 1) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) on a Novex 4-20% Tris-Glycine Mini Protein Gel under reducing and/or non-reducing conditions, followed by staining with Coomassie Brilliant Blue, compared to Benchmark Protein Ladder (ThermoFisher, 10747-012), or 2) run on PerkinElmer Caliper LifeSciences LabChip GXII according to manufacturer's suggested guidelines.

2.7.3. Analytical Size Exclusion Chromatography (SEC)

All final purification pools, and some selected fractions, were analyzed by anaSEC using a GE 5/150 Superdex200 Increase column (GE 28-9909-45) connected to the Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system at 0.2mL/minute in SEC Loading Buffer (100mM NaPO4, 20mM NaCl, 0.05%NaN_3, pH6.8).

Chapter III

Results

3.1 Fusion proteins

A number of fusion proteins were designed, expressed, and purified for use in all experimental work. Three constructs (Figure 3.1) were used as proof of concept models, and were involved in various aspects of the work, including antibody screening, binding studies, and for testing of the three proposed immunoaffinity purification methods.

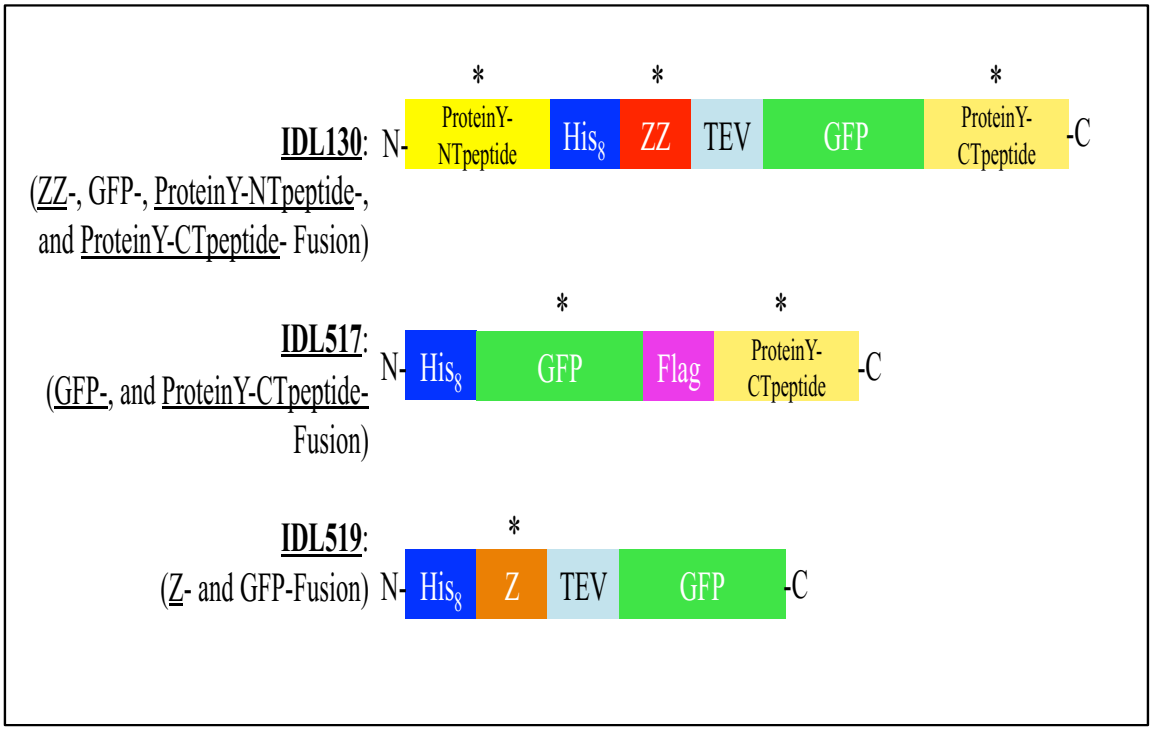


Figure 3.1. Schematic representation of proof of concept fusion-protein constructs used in this study.

Fusion protein IDL130 (MW 44,258Da) was used in the ProteinY-peptide-fusion and the ZZ-fusion projects. IDL517 (MW 38,142Da) was used in the GFP-fusion and the

ProteinY-peptide-fusion projects. IDL519 (MW 37,649Da) was used in the ZZ-fusion project.

A GFP-fusion tag was included in all constructs, not only so that it could be targeted in the cAbGFPmutant-purification system, but also to allow visualization of the proteins of interest, making it easier to determine where proteins were located in various steps of the purification. Additionally, each construct included a His-tag to be used in an alternative purification (Nickel IMAC) method to the test purification method.

3.1.1. Expression and Purification

The fusion proteins were stably expressed in CHO-DG44 cells, and expression levels were determined by Octet Concentration Analysis, as described in *Materials and Methods (Protein Expression: Fusion Proteins)*. All three proteins expressed well (100mg/L or higher). A portion of each fusion protein was purified on an AKTA via a nickel IMAC (Ni-NTA or HisTrap Excel) resin, resulting in 25-35mg each of Nickel-purified material. Briefly, the samples were loaded on a column equilibrated with buffer containing 20mM Imidazole and eluted with a buffer containing 500mM Imidazole. IDL130 was eluted in steps, with a 250mM Imidazole elution prior to the 500mM elution step, which resulted in two peaks. The second peak was of higher purity and was used for these studies. An AKTA chromatogram of each Nickel-IMAC purification can be found in Appendix 1.

3.1.2. Characterization of Nickel-purified Fusion Proteins

Once the proof of concept fusion constructs had been expressed and Nickel-purified, each was quantified by UV/Vis, with concentration determined by using the

calculated extinction coefficient for 280nm (Table 3.1).

Table 3.1. Concentration of Nickel-purified Proof of Concept Fusion-Proteins.

Construct (Molecular Weight)	Extinction Coefficient	A280	Calculated concentration (mg/mL); (volume)	Total mg
IDL130 (44.3 kDa)	0.60	1.97	3.28mg/mL; (9mL)	30
IDL517 (38.1 kDa)	0.85	1.49	1.75mg/mL; (20mL)	35
IDL519 (37.6 kDa)	0.75	0.93	1.24mg/mL; (20mL)	25

Concentration (mg/mL) of Ni-purified fusion proteins based on extinction coefficients.

The Nickel IMAC-purified fusion proteins were also characterized by analytical SEC and SDS-PAGE (Figures 3.2, 3.3, and 3.4). SDS-Page indicates pure samples of all three, and analytical SEC indicates IDL130 and IDL517 are low aggregate, but IDL519 has two peaks, the earlier of which may be aggregate or dimerization of the protein.

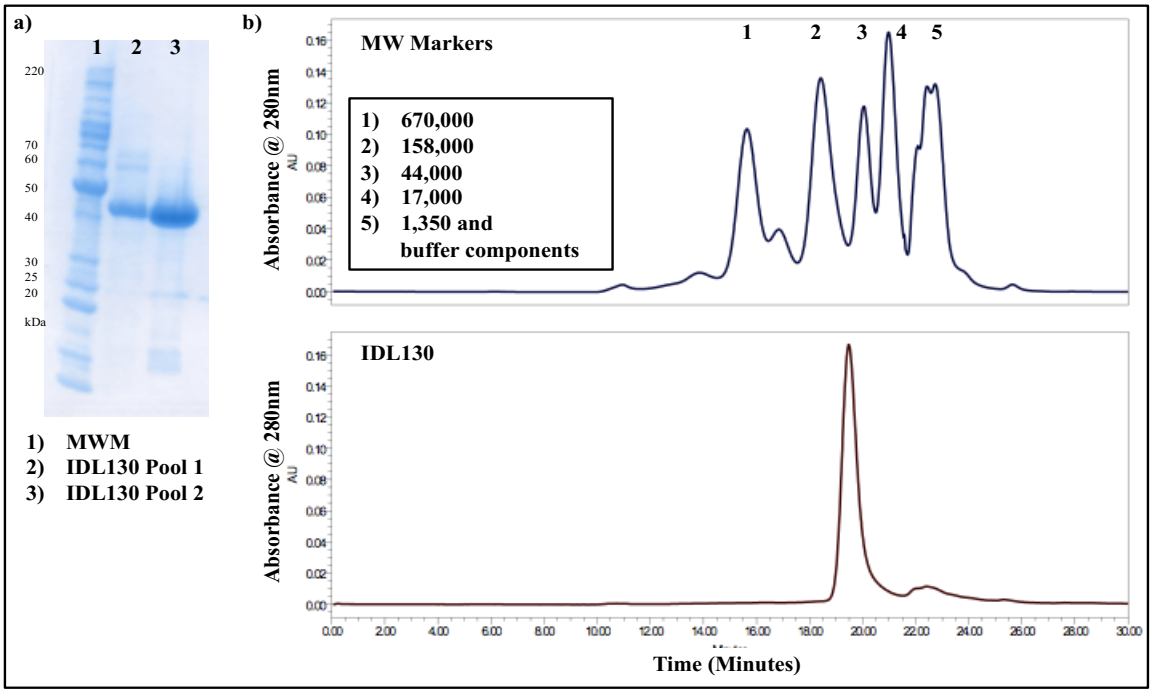


Figure 3.2. Characterization of Nickel-purified **IDL130** (ZZ-, GFP-, CTpeptide-, NTpeptide-fusion protein) by **a** SDS-Page (non-reduced) and **b** analytical SEC. Pool 2 was more pure than pool 1, and was used for thesis work.

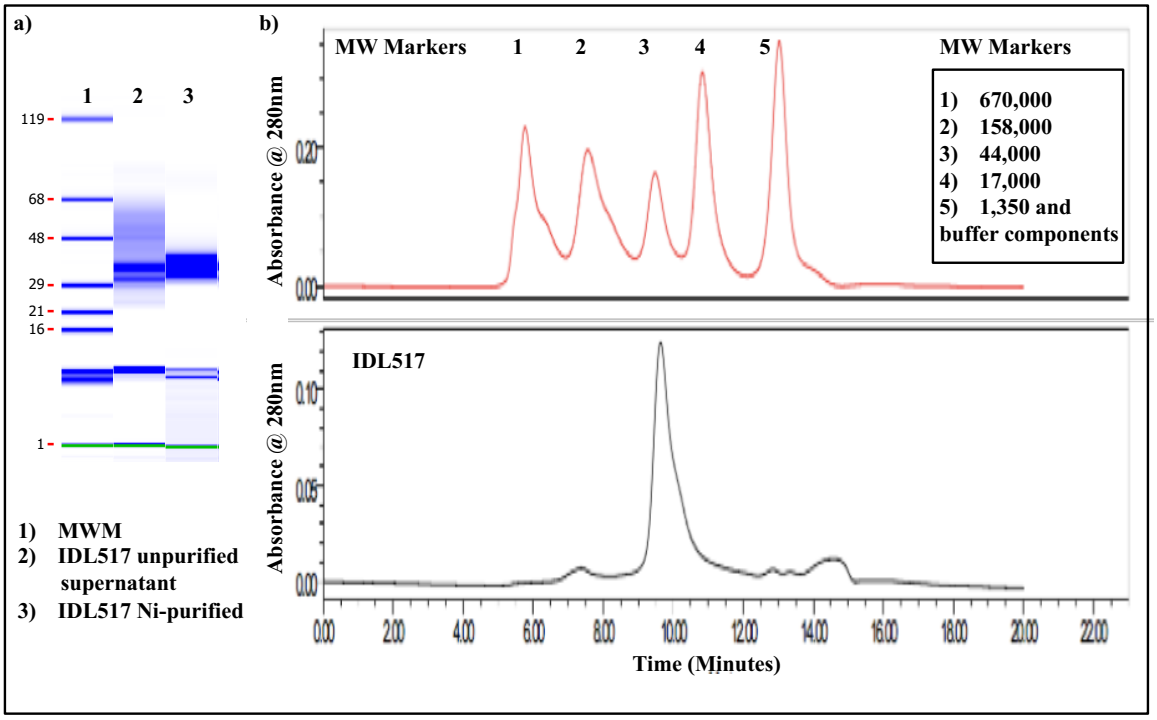


Figure 3.3. Characterization of Nickel-purified **IDL1517** (GFP-, ProteinY-CTpeptide-fusion protein) by **a** Gel-CHIP analysis (non-reduced) and **b** analytical SEC.

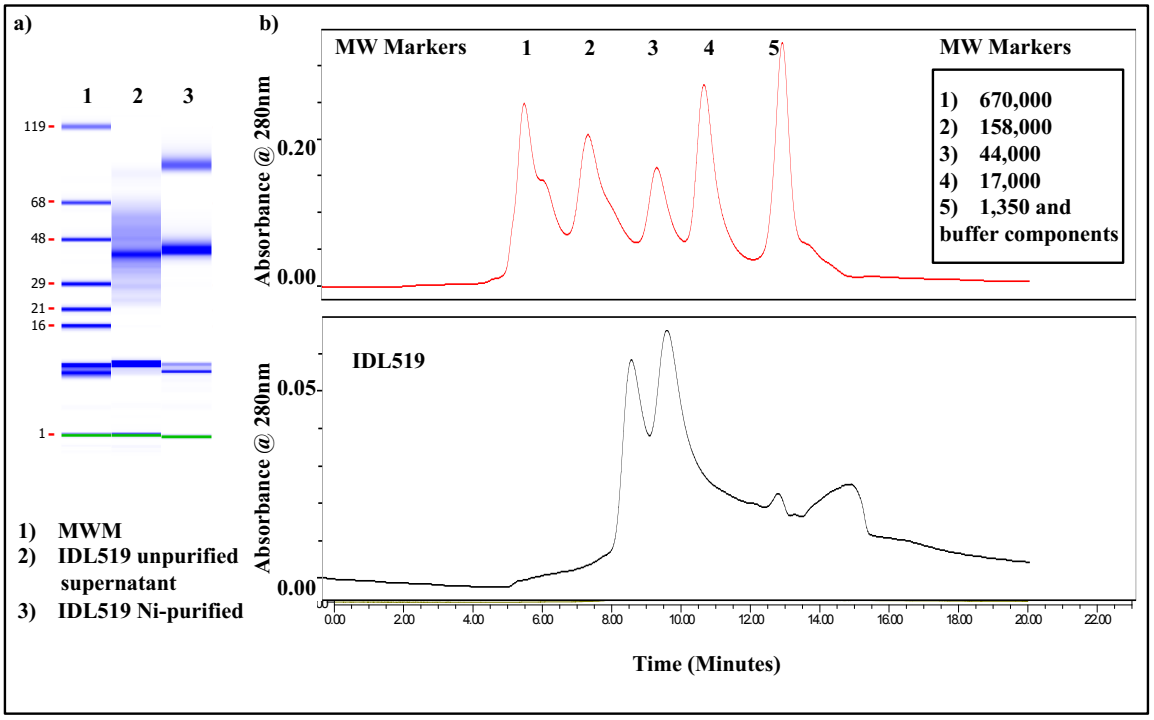


Figure 3.4. Characterization of Nickel-purified **IDL1519** (Z-fusion) by **a** Gel-CHIP analysis (non-reduced) and **b** analytical SEC.

3.2. Immunoaffinity Purification Methods

For each of the three proposed immunoaffinity purification methods, a number of experimental steps were performed. They were performed to determine the proper immunoaffinity antibody for binding the fusion-tag, to optimize the conditions for purification of fusion-proteins, and to properly analyze the method in comparison to a well-established purification method.

3.2.1 ProteinY-peptide-fusion purification

While the Flag™-tag is a common tag used in recombinant protein purification, it can be difficult to elute Flag™-tag recombinant protein fusions from anti-Flag immunoaffinity resins. Elution often requires either a low pH elution, which, as previously stated, can be detrimental to certain proteins, or the more typically used competitive elution by excess Flag™-peptide. Additionally, anti-Flag antibodies are expensive, and although their affinity for Flag™-tag is high, they have a low binding capacity for Flag™-tag, which can make large-scale purification of such fusion proteins costly (Einhauer & Jungbauer, 2001; Waugh, 2005). To address the issues of expense, in-house antibodies raised against two peptides from protein Y were investigated for their utility in immunoaffinity purification of fusion proteins. Like the FLAG™-tag strategy, protein Y-peptides were fused to recombinant proteins. Since the mAbs targeting the peptide sequences are available and able to be produced in-house, this greatly reduces the costs often associated with purification of Flag™-tagged proteins. Additionally, like with a Flag™-tag, the peptide itself can be used for competitive elution of the peptide-fusion protein, enabling for a neutral pH location.

3.2.1.1 ProteinY-peptide antibody selection. In a previous antibody campaign at Biogen,

antibodies were raised against two peptides from protein Y, (designated as such due to confidentiality). The two peptides each consisted of nine amino acid residues, and were located at either end of the ProteinY sequence, therefore are designated ProteinY-NTpeptide (N-terminal) and ProteinY-CTpeptide (C-terminal). From this campaign, six antibodies were developed that targeted the two peptide sequences (three were specific for CTpeptide, and three were specific for NTpeptide). In order to identify the best antibody candidate for use in column format, a number of analytical techniques were used to screen the antibodies, including ELISA, and mini bead-purification tests.

3.2.1.1.1. ELISA analysis of antibodies. ELISA was used to assess the binding of the antibodies to the peptides they were raised against (Figure 3.5).

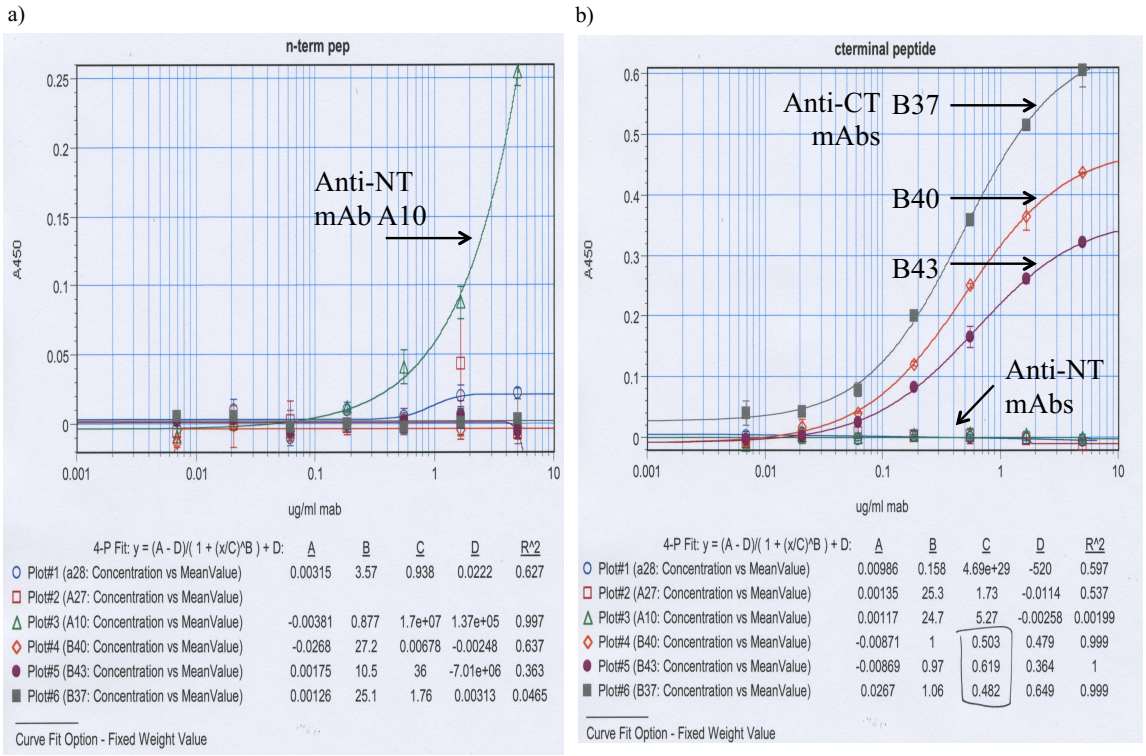


Figure 3.5. ELISA curve-fits of six anti-proteinY-peptide mAbs (3 anti-proteinY-CT mAbs: B37, B40, B43; and 3 anti-ProteinY-NT mAbs: A10, A27, and A28) on a plate coated with **a** ProteinY-NTpeptide, and **b** ProteinY-CTpeptide.

Plates were coated with peptides and antibody dilutions were tested as described in *Materials and Methods (Antibody screening: ELISA)*. No significant binding to either peptide was observed by the anti-NTpeptide-mAbs. Binding to CTpeptides was observed for all three anti-CTpeptide-mAbs, and no binding to NTpeptides by anti-CTpeptide-mAbs was observed. Based on these results, the anti-CTpeptide antibodies are suitable for further investigation as immunoaffinity antibodies. Although ELISA results indicate weak binding of anti-NTpeptide mAbs to either peptide, they were included in column format tests because they had potential to be useful in the column binding conditions.

3.2.1.1.2 Peptide-fusion test purification via antibody-Protein A resin. After initial binding analysis by ELISA, the antibodies were tested in two different column formats. One format was using the antibodies bound to protein A and the second was using the antibodies covalently coupled to NHS-sepharose. The initial format, Protein A bead analysis, was used to quickly determine if these mAbs might be useful for protein purification using peptide elution. Briefly, the antibodies were mixed with Protein A resin, as described in Materials and Methods (Antibody Screening: Protein A-bound antibody resin analysis), and washed before mixing with fusion protein IDL130, containing both NT- and CT-peptide, as well as a GFP-tag, the visibility of which allowed for easy determination of the recombinant protein's location during the test. This was followed by a wash with PBS then test elution with an excess of synthetic NTpeptide, which acted as a competitor for the anti-peptide mAb. Following peptide elution, a low pH elution (with 25mM $NaPO_4$, 100mM NaCl, pH2.8) was performed to remove any non-eluted fusion protein, and also elute the antibody from the Protein A resin. Each step was sampled and analyzed by gel to determine location of peptide-fusion IDL130. When using any of the three anti-ProteinY-NTpeptide mAbs (mAbs A10, A27,

and A28), fusion protein was seen in the non-bound flow through only, and only antibody was in the sample of resin following the fusion protein load (Figure 3.6a, mAb A10 shown). In the peptide elution fractions, no fusion protein was visualized, and in the resin sample following peptide elution, as well as the low pH wash, only antibody was visible. Fusion protein IDL130 did not bind to any of the anti-ProteinY-NTpeptide mAbs, and these antibodies would not be useful for immunoaffinity purification. In contrast, in all three tests using the anti-ProteinY-CTpeptide mAbs (mAbs B37, B40, and B43), IDL130 was seen in the sample of resin following the load, as well as in both peptide elution fractions (Figure 3.6b, mAb B40 shown). The samples of resin following peptide elution, and of the low pH wash, each contained only anti-ProteinY-CTpeptide mAb. Fusion protein IDL130 did bind to the anti-ProteinY-CTpeptide mAbs, and was eluted with the peptide elution. Of the three anti-Protein Y-CT peptide mAbs, B40 was chosen as the candidate to move forward, simply because of the quantities of antibody available.

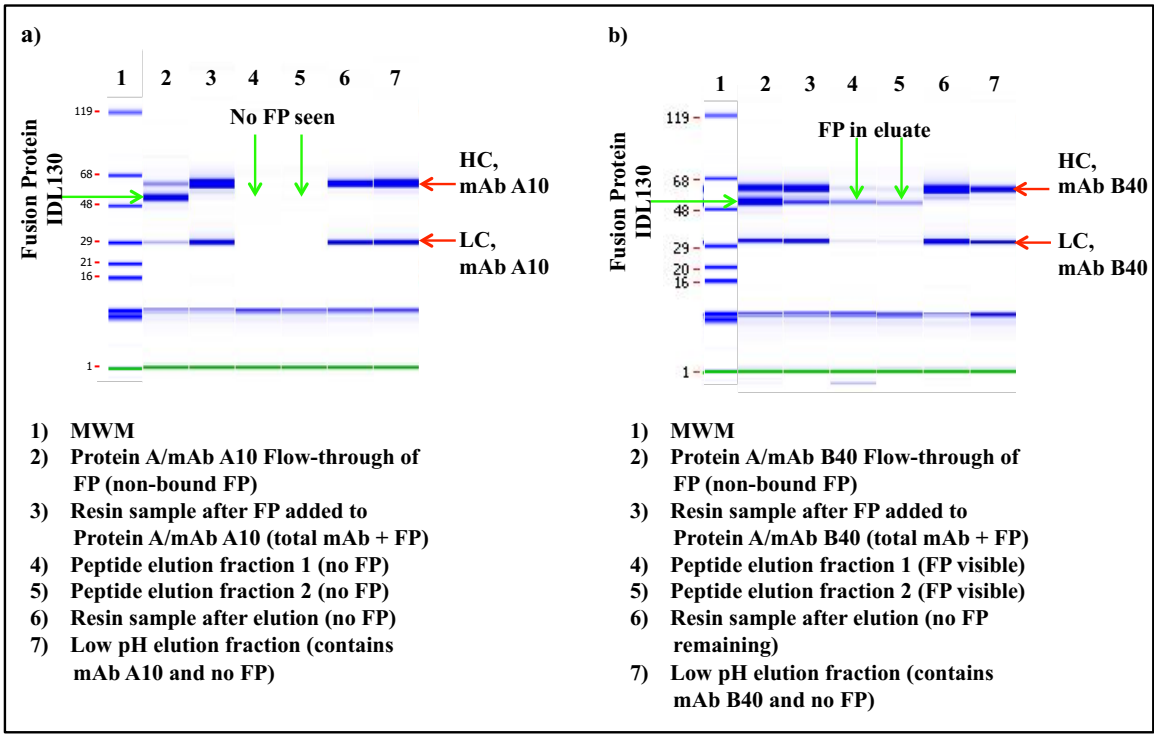


Figure 3.6. On-column anti-ProteinY-peptide antibody test, Format 1. Gel-CHIP analysis of small-scale purification test of **ProteinY-peptide-fusion IDL130** on Protein A resin bound with **a** ProteinY-NTpeptide mAb A10, or **b** ProteinY-CTpeptide mAb B40.

3.2.1.2. Purification via anti-ProteinY-CTpeptide mAb immunoaffinity column. To test the ability of the chosen anti-ProteinY-CTpeptide mAb, mAb B40, to purify ProY-CTpeptide-fusion protein in a more typical column-format, mAb B40 was covalently immobilized to NHS-activated agarose. The resin was prepared as described in *Materials and Methods (Covalent Immobilization of Antibodies to NHS-Activated Agarose)*. In the SDS-Page analysis of the coupling steps, no antibody was observed in the coupling flow-through. All of the antibody (10mg) that was mixed with resin was successfully bound to it.

Following coupling, 1.0mL of the mAb B40 immunoaffinity resin was transferred into a small column and used on an AKTA to purify CTpeptide-fusion protein (IDL517)

supernatant. UV/Vis was recorded at 280nm and 495nm throughout the run to follow the protein (Figure 3.7). The protein was bound to the column in PBS and eluted in PBS with an excess of synthetic ProteinY-CTpeptide (10mM). Media components, and possibly protein loaded in excess of column capacity, resulted in the 280nm flow-through peak early in the chromatogram. The elution step resulted in a single peak, which was collected in fractions, and each fraction was analyzed by gel before pooling. To determine if any protein remained on the column following elution with excess CTpeptide, the column was further eluted with low pH buffer (25mM NaPO4, 100mM NaCl, pH2.8). No peak was observed in the follow-up low pH elution. The elution with excess CTpeptide was sufficient.

The recovered pool of CTpeptide-fusion IDL517 consisted of 0.50mg. On a 1.0mL ProteinY-CTpeptide mAb B40 immunoaffinity column, the capacity for CTpeptide-fusion protein per mL of resin is 0.5mg/mL.

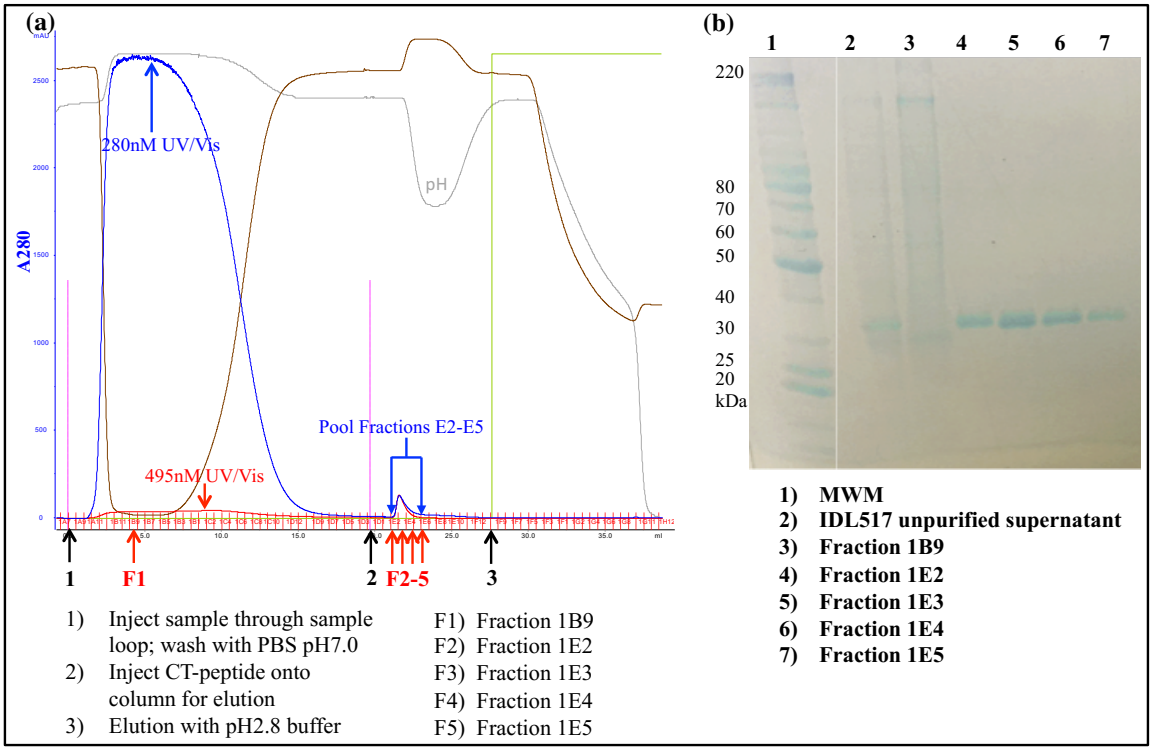


Figure 3.7. Purification of **ProteinY-CTpeptide-fusion IDL517** supernatant with anti-ProteinY-CTpeptide-(mAb B40)-NHS immunoaffinity column, illustrated by **a** chromatogram, **b** SDS-Page of (2) unpurified IDL517 supernatant, (3) fraction 1B9, and (4-7) CTpeptide-elution fractions.

3.2.1.3. Characterization of immunoaffinity-purified peptide-fusion and comparison to Nickel-purification. The final sample was compared to the Nickel-purified samples to determine if the anti-ProteinY-CTpeptide mAb affinity method of purifying CTpeptide-fusion protein results in a sample as pure as a standard Nickel-purification of the same sample, and to compare capacity and recovery as well. To compare, samples were analyzed by gel and by analytical SEC (Figure 3.8).

The profile by gel at least matched that of the Nickel-purified profile, and may even be slightly more pure. The profile by analytical SEC had more low-molecular weight contaminants than the Nickel-purified form. The low-molecular weight contaminants are likely excess peptide still present in the sample. The recovery of

CTpeptide-fusion IDL517 via Nickel-purification on a 5mL column was 7mg/mL of resin. The recovery of the same construct via anti-ProteinY-CTpeptide (mAb B40)-NHS purification was 0.5mg/mL of resin. Though significantly lower than NiNTA, this capacity is similar to FLAG.

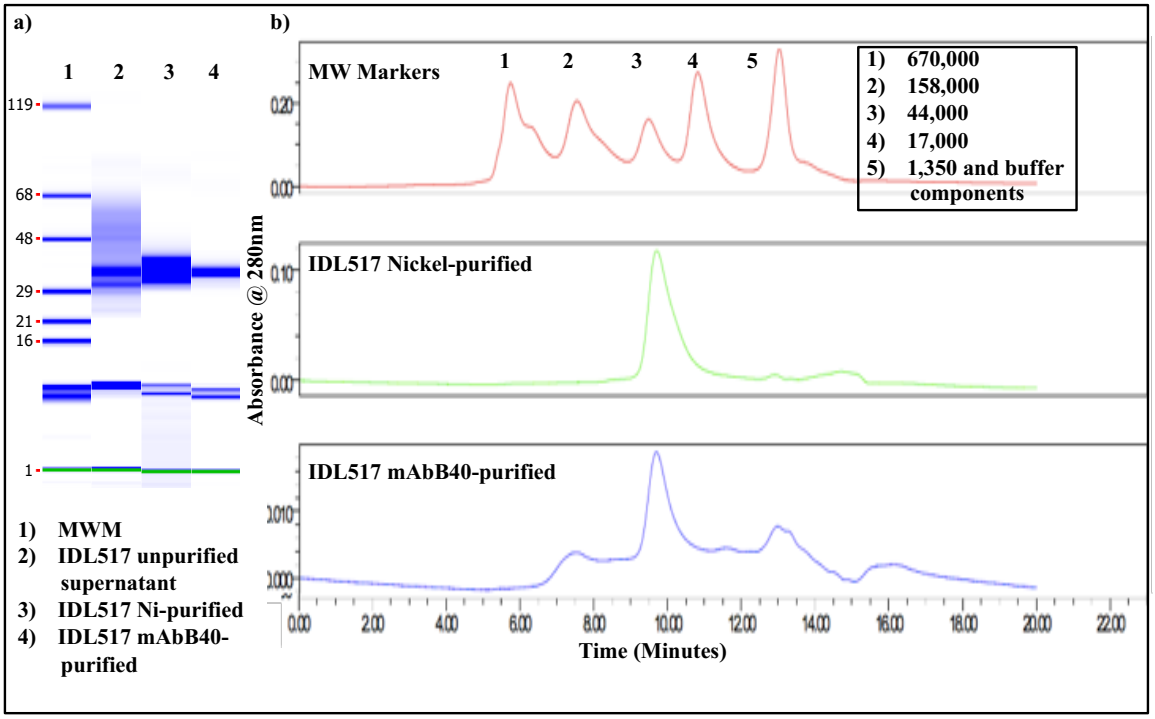


Figure 3.8. Characterization comparison of Nickel-purification of **IDL1517** to anti-ProteinY-CTpeptide-(mAb B40)-NHS immunoaffinity purification of same, by **a** Gel-CHIP analysis (non-reduced) and **b** analytical SEC.

3.2.2. GFP-fusion Purification

While GFP-fusions exist and are used as a purification tool, the extremely high affinity that anti-GFP antibodies have for GFP makes it very difficult to separate the antibody from the protein, and impossible in anything but extreme pH elution conditions, which have negative effects on GFP, a pH-sensitive protein (Rothbauer et al., 2008;

Patterson et al., 1997; Tsien, 1998). In order to address this issue, a number of cAbGFPmutants were designed and expressed in an attempt to decrease the avidity that cAbGFP has for GFP in a neutral pH range. GFP was fused to recombinant protein so that the cAbGFPmutants could be investigated for their utility in immunoaffinity purification of the GFP-fusion proteins.

3.2.2.1 cAbGFPmutant antibody design and selection. The mutations were designed based on the crystal structure and included single and multiple point mutations. All mutations involved replacing an amino acid residue with a histidine residue, in an attempt to adjust the charge in the antigen-binding region of the antibody in order to convey pH-dependent binding to GFP. Due to potential pending patents, the exact location of the mutations cannot be specified.

3.2.2.1.1 Octet Kinetic Analysis. Following construction of the mutant cAbGFP antibodies, they were screened for pH-dependent binding to GFP at pH 5.5 and pH 9.5. Plasmid DNA was generated for 36 cAbGFPmutant constructs, and a 5mL small-scale expression was performed to recover a small amount of antibody for assessing GFP-binding by Octet Kinetic Analysis. A complete list of constructs, each with its number of mutations, theoretical molecular weight, theoretical pI, and raw octet kinetic analysis interpretation, can be found in Appendix 2.

Briefly, Protein A sensor tips were equilibrated in PBS, loaded with cAbGFPmutant, dipped in PBS, then dipped into wells containing GFP in buffer at either pH5.5, pH7.0, pH9.0, or pH9.5. Most mutants (27 of 36) had little or no binding of GFP at pH9.0 or pH9.5. Two had good binding at pH5.5, but with only slight pH sensitivity (less binding) at pH9.5. Two had low binding overall, but displayed the desired pH sensitivity (higher binding at pH 5.5). Five of the 36 displayed good binding at pH5.5,

with decreased binding at pH9.5. Based on these GFP-binding results, eight candidates were selected for further investigation, including the two with low binding overall, but pH sensitivity, two with no binding at pH9.5, and four with good binding at pH5.5 and significant pH-sensitivity at pH9.5 (Table 3.2).

Table 3.2. Eight mutant cAbGFPs selected for further analysis.

Construct	cAbGFP-hIgG1 mutation(s)	Octet Kinetic Analysis of GFP-binding at pH5.5, pH9.0, and/or pH9.5	Theoretical MW (kDa)	Theoretical pI
IDL202	1 H	No binding at pH9.5	38195	7.69
IDL206	2 H	No binding at pH9.5	38137	8.07
IDL649	2 H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38218	6.82
IDL650	3 H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38199	6.59
IDL652	**3 H**	**Good binding at pH5.5, significant pH sensitivity at pH9.5**	**38139**	**6.59**
IDL653	4 H	Low binding overall, pH sensitive	38090	6.64
IDL655	3 H	Low binding overall, pH sensitive	38090	6.64
IDL656	3 H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38159	6.82

Eight mutant cAbGFP constructs selected for further analysis, determined by interpretation of Octet Kinetic Analysis evaluation results.

3.2.2.1.2 Expression and Purification. The eight selected cAbGFP mutants were transiently expressed in CHO at medium scale (200 mL) to obtain sufficient amounts of antibody for further studies. The transient CHO-expressed antibodies were harvested 11 days post-transfection, and the concentration of each was analyzed by Octet Concentration Analysis, in order to determine appropriate column sizes to use in purification steps. All the transients expressed well, at nearly 100mg/L or higher. Each mutant was purified on a 5mL Protein A column, and fractions were pooled according to absorbance. Between 12-35mg was recovered of each, and the neutralized, filtered pools

were analyzed by gel for purity (Appendix 3).

3.2.2.1.3. GFP-fusion test purification via antibody-Protein A resin. After narrowing the cAbGFPmutants down to eight candidates, small-scale GFP-purification tests, as described in Materials and Methods (Antibody Screening: Protein A-bound antibody resin analysis), were performed with the mutants to choose a single candidate to serve as the immunoaffinity antibody for GFP-fusion purification. Briefly, each of the eight mutants was mixed with Protein A beads to simulate an immunoaffinity column format. A ninth tube of Protein A resin contained no mutant and served as a negative control. The Protein A-cAbGFP mixtures were washed and mixed with GFP-fusion IDL517. The GFP allowed for easy visual determination of the recombinant protein's location throughout the tests. Following the mixing of Protein A-bound cAbGFP with GFP-fusion IDL517, green was very obviously observed in the flow-through of the negative control (Figure 3.9, a, sample I), and was observed slightly in other samples. The same result was true of the washes. Following elution with 20mM BisTris-Propane pH9.0, 150mM NaCl, three samples had very little to no green visibly remaining on the resin: IDL650, IDL652, and IDL653.

A second test was run with these three antibodies, however they were eluted with BisTris-Propane pH9.5, instead of pH9.0, to see if a more obvious elution occurred. After one elution fraction, green was observed in the elution fraction of two of the samples, and little green remained visible on the resin (Figure 3.9, b-c). Mutant IDL652, which contains three mutations to histidine, was chosen as the candidate to move forward with, because its results looked slightly better, and our purified protein stock of it was slightly greater.

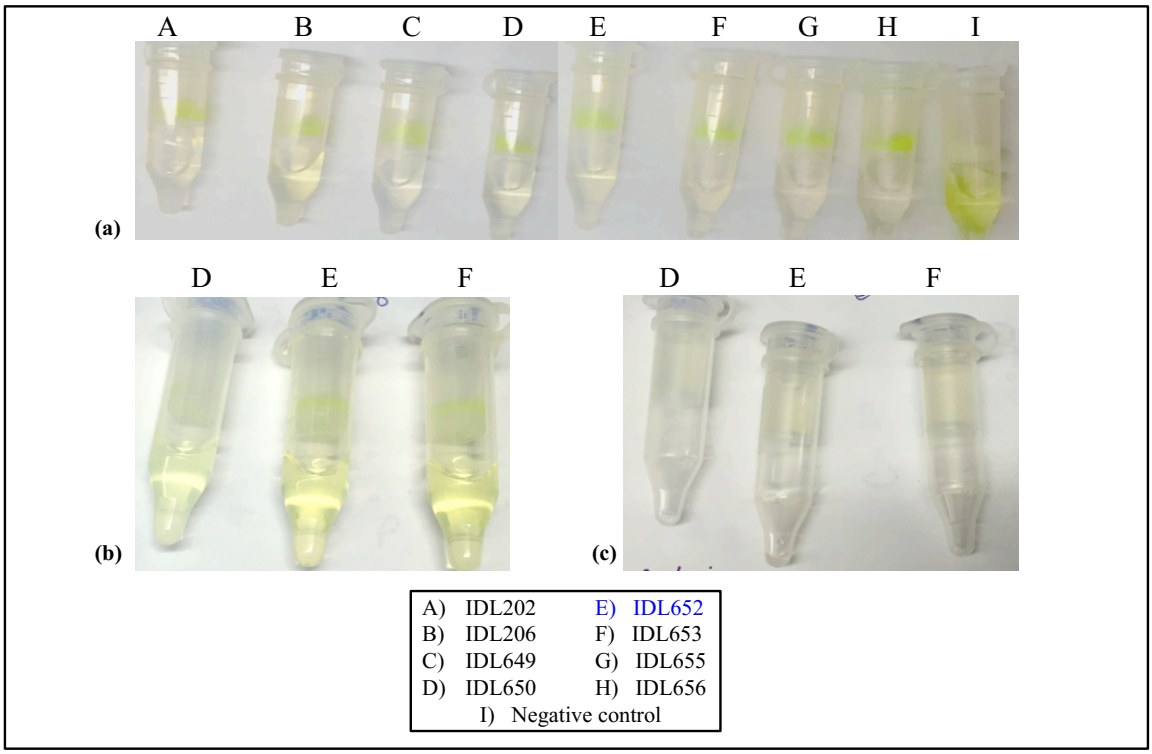


Figure 3.9. Visualization of small-scale on-column purification testing of cAbGFPmutants. **a** GFP flow-through of all 8 cAbGFPmutant antibody-resins (and one negative control), **b** pH9.5 elution for three antibodies, **c** resin following pH9.5 elution for three antibodies.

3.2.2.2. Purification via cAbGFPmutant immunoaffinity column. To test the ability of cAbGFPmutant IDL652 to purify GFP-fusion protein in a more typical column-format, cAbGFPmutant IDL652 was covalently immobilized to NHS-activated agarose. The resin was prepared as described in *Materials and Methods (Covalent Immobilization of Antibodies to NHS-Activated Agarose)*. In the SDS-Page analysis of the coupling steps, no antibody was observed in the coupling flow-through. All of the antibody (4.6mg) that was mixed with resin was successfully bound to it.

Following coupling, 0.5mL of the cAbGFPmutant immunoaffinity resin was transferred into a small column and used on an AKTA to purify a sample of GFP-fusion protein (IDL517) supernatant. UV/Vis was recorded at 280nm and 495nm throughout the

run to follow the protein (Figure 3.10). The protein was bound to the column in PBS pH 5.5 and eluted in 20mM BisTris-Propane, 150mM NaCl, pH9.5. Media components, and possibly protein loaded in excess of column capacity, resulted in the 280nm flow-through peak early in the chromatogram. The elution resulted in a single peak, which was collected in fractions, and each fraction was analyzed by gel before pooling. To determine if any protein remained on the column following pH9.5 elution, the column was further eluted with low pH buffer (25mM NaPO4, 100mM NaCl, pH2.8) No peak was observed in the follow-up low pH elution. The elution with pH 9.5 buffer was sufficient.

The recovered pool of GFP-fusion IDL517 consisted of 1.65mg. On a 0.5mL cAbGFPmutant immunoaffinity column, the capacity for GFP-protein per mL of resin is 3.3mg/mL.

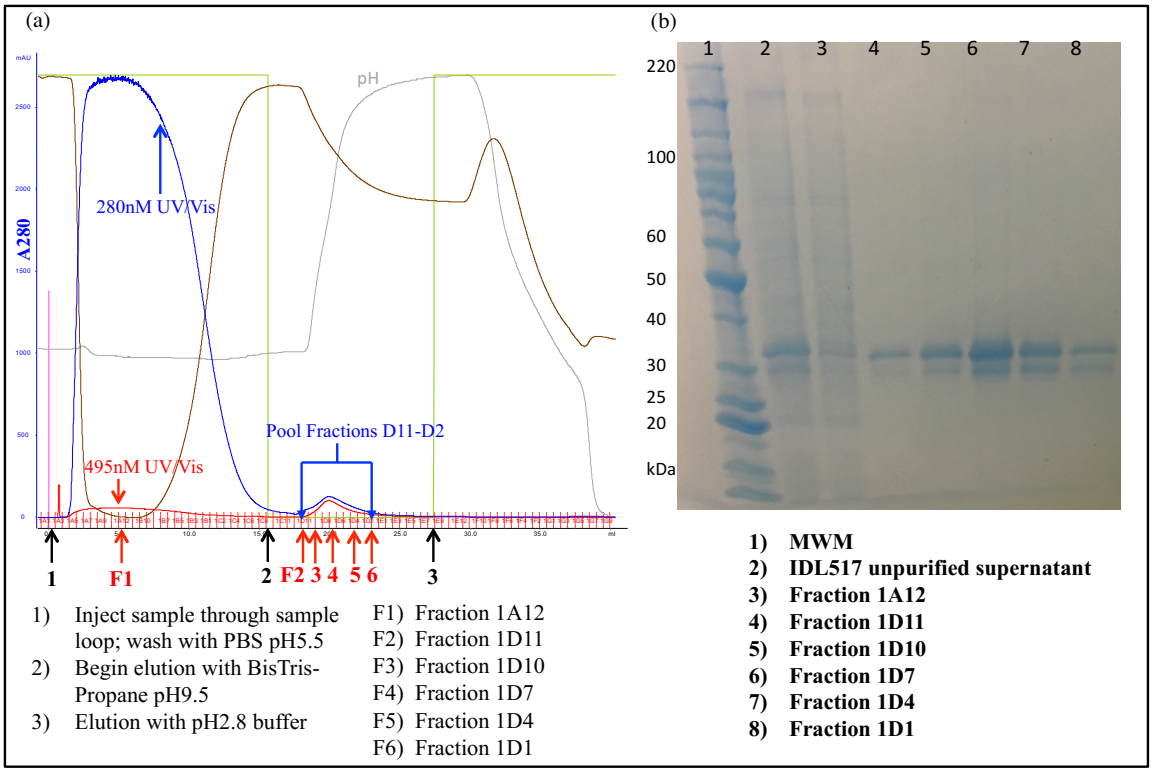


Figure 3.10. Purification of **GFP-fusion IDL517** supernatant with cAbGFPmutant IDL652-NHS immunoaffinity column, illustrated by **a** chromatogram and **b** SDS-Page of (2) unpurified IDL517 supernatant, (3) fraction 1A12, and (4-8) pH9.5 elution peak fractions.

3.2.2.3. Characterization of immunoaffinity-purified GFP-fusion and comparison to Nickel-purification. The final sample was compared to the Nickel-purified sample to determine if the cAbGFPmutant affinity method of purifying GFP-fusion protein results in a sample as pure as a standard Nickel-purification of the same sample, and to compare capacity and recovery as well. To compare the purification methods, samples were analyzed by gel and by analytical SEC (Figure 3.11).

The profile by gel matched that of the Nickel-purified profile. The profile by analytical SEC had more low-molecular weight contaminants than the Nickel-purified form when scanned at 280nm, but was nearly identical to the Nickel-purified profile when scanned at 495nm. The recovery of GFP-fusion IDL517 via Nickel-purification

was 7mg/mL of resin (on a 5mL column). The recovery of the same construct via cAbGFPmutant (IDL652)-NHS purification was 3.3mg/mL of resin.

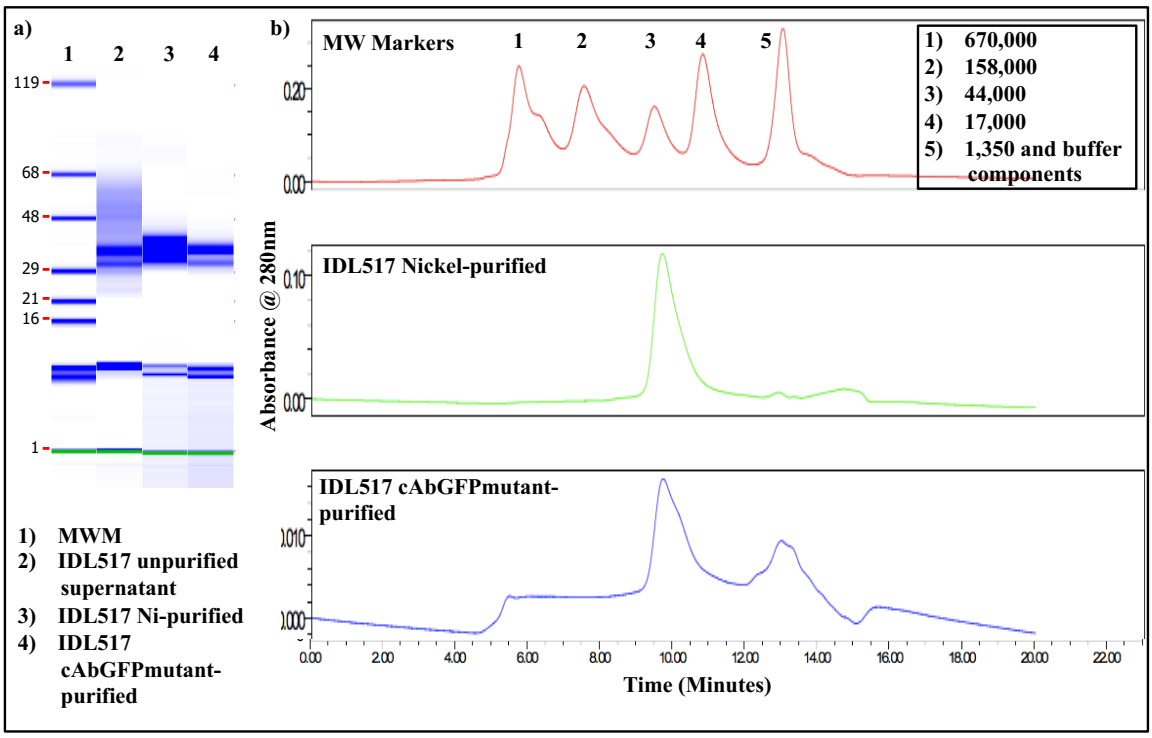


Figure 3.11. Characterization comparison of Nickel-purification of **IDL1517** to cAbGFPmutant(IDL652)-NHS immunoaffinity purification of same, by **a** Gel-CHIP analysis (non-reduced), and **b** analytical SEC.

3.2.3. ZZ-fusion Purification

The purification of Fc-bearing proteins using Protein A typically requires an elution step with a low pH buffer, such as a phosphate buffer at pH2.8, to efficiently separate the protein of interest from the affinity column. However the affinity between Protein A and murine IgG1 Fc is weak and binding to a protein A resin requires high pH (9.0) and high salt (NaCl) concentrations (Ey et al., 1978; Malm, 1987). The murine IgG1 Fc does not require the typical low pH for Protein A elution following the high pH/high

NaCl binding, but can rather be eluted gently by reducing the pH to 7 and reducing the NaCl concentration to 150mM. These unique binding characteristics between protein A and murine IgG1 Fc were investigated as a novel purification approach with gentle elution. The Z domain is an engineered Fc binding domain derived from the B domain of Protein A (see *1.4.3. Using the ZZ-tag as a murine IgG1 Affinity Tag*). A fusion protein containing tandem Z domain, termed ZZ-tag, was initially used for these studies (IDL130). Preliminary results suggested that the ZZ and murine IgG1-Fc affinity might be too high for use in a column format so a protein containing only one Z domain was also tested (IDL519). For this purification approach, the Z and ZZ-tagged fusion proteins were purified on a murine IgG1 affinity column, with the binding step occurring in high pH (9.0), high NaCl (3M) conditions, and then eluted with PBS, which has reduced pH (7.0) and NaCl (150mM) conditions.

3.2.3.1. murine IgG1 antibody selection. To ensure that there are no antibody-specific effects, a panel of in-house murine IgG1s was tested. In previous antibody campaigns at Biogen, antibodies of various isotypes were raised against numerous proteins, making available numerous in-house murine IgG1 antibodies. Three murine IgG1 antibodies were chosen and designated anti-R murine IgG1, anti-H murine IgG1 (WT), and anti-H murine IgG1 (agly). In addition, an anti-H mIgG2a was used as a control, as it would have greater affinity for ZZ than murine IgG1, so could serve as a positive control for binding. Due to its affinity for ZZ, it would necessitate a low pH elution step for separation purposes, so could also serve as a negative control in the elution step. In order to identify the best murine IgG1 candidate for use in column format, Octet Kinetic Analysis was performed, followed by a mini bead-purification test.

3.2.3.1.1. Octet Kinetic Analysis. Briefly, anti-murineFc (AMC) sensor tips were equilibrated in PBS, each loaded with a various murine IgG1 antibody or a mIgG2a positive control, equilibrated in PBS or high-NaCl, high-pH buffer, then dipped into wells containing either ZZ-fusion protein IDL130, or in wells containing Z-fusion IDL519, and lastly dissociated in PBS. A second analysis was performed using ProA and ProG sensor tips, and only the mIgG2a, to determine if the binding to ZZ or Z occurred in the same location as the tip-binding.

Attempts were made to screen the murine IgG1s for binding and dissociation at pH7 via Octet Kinetics. However, the results were difficult to interpret, likely due to the unusual and extreme buffer conditions (high pH, high NaCl versus normal PBS). This complexity of components, along with a limited availability of tip formats, made this test unusable. The inconclusive results could not be assumed to translate correspondingly to an on-column format, so the binding capability was tested directly in an on-column format.

3.2.3.1.2. ZZ- and Z-fusion on-column murine IgG1 binding analysis. To test the ZZ-binding capacity and purification ability of the chosen murine IgG1 antibodies, each of the three was covalently immobilized to NHS-activated agarose, along with the mIgG2a control. The resins were prepared as described in Materials and Methods (Covalent Immobilization of Antibodies to NHS-Activated Agarose). In the SDS-Page analysis of the coupling steps, no antibody was observed in the coupling flow-through. For each resin/antibody combination, all of the antibody (7.1–9.9mg) that was mixed with resin was successfully bound to it.

Following coupling, 1.0mL of each murine IgG1 and mIgG2a immunoaffinity resin was used to purify the fusions in a gravity column. ZZ-fusion IDL130 supernatant

and Z-fusion IDL519 (Nickel-purified sample) were loaded and purified on each column, as described in *Materials and Methods (Immunoaffinity Purifications of Fusion Proteins: ZZ-fusion)*. All of the flow-through, wash, and elution fractions were analyzed by UV/Vis on Xpose, to determine binding capacity and purification ability (Table 3.3).

Table 3.3. Concentration (mg/mL) of various fractions during purification of ZZ-fusion and Z-fusion constructs on murine IgG1 and murine IgG2a immunoaffinity columns.

Fusion tested	IDL130 (ZZ)	IDL130 (ZZ)	IDL130 (ZZ)	IDL519 (Z)	IDL519 (Z)	IDL519 (Z)	IDL519 (Z)
Antibody column	Anti-R mIgG1	Anti-H WT mIgG1	Anti-H agly mIgG1	Anti-R mIgG1	Anti-H WT mIgG1	Anti-H agly mIgG1	Anti-H mIgG2a
Antibody coupled/	7.1mg	9.9mg	9.4mg	9.0mg	9.5mg	9.3mg	9.6mg
Fusion loaded	2.0mg	2.0mg	2.0mg	2.43mg	2.43mg	2.43mg	2.43mg
Fusion bound	1.84mg	1.34mg	1.74mg	0.57mg	0.08mg	0.25mg	1.70mg
Fusion eluted	0.28mg	0.20mg	0.40mg	0.13mg	0.07mg	0.15mg	0.04mg
% Eluted of bound	15%	15%	23%	23%	82%*	60%	2%

Binding and eluting capabilities of various murine IgG1-immunoaffinity resins, for ZZ-fusion (supernatant) and Z-fusion (already-purified) fusion proteins.

The results show that very little (less than 0.6mg) of the Z-fusion protein actually bound to murine IgG1 columns, and significantly less so in comparison to the ZZ-fusion, as expected, based on the avidity effect of the ZZ. Of the material that did bind to the resin in high NaCl, high pH buffer, less than 0.28mg of the 2-2.4mg load was eluted from each fusion/mIgG1 combination (except in one case, 0.4mg was eluted), and the recovery (% eluted of bound) was less than 25% in almost all cases. In the two cases (both with the Z-fusion) that recovery was higher than 25%, the binding capacity was less than 0.25mg/mL of resin, which is extremely low. The murine IgG2a column bound 1.7mg of

Z-fusion, but nearly nothing was eluted in PBS.

Because the results for both fusions and for multiple murine IgG1 antibodies were so minimal, no further work was performed for this method, and it was determined that it would not be a useful purification tool.

3.3. Final Immunoaffinity Purification Method Comparison

Following all completed purification work, the two purification methods that successfully yielded purified protein were compared to Nickel-purified material. Because fusion protein IDL517 contained a GFP-fusion tag, a ProteinY-CTpeptide-fusion tag, and a His-tag, it could be purified by both purification methods, as well as a Nickel-purification control method. Thus, the purity and aggregation of the results of each method can be directly compared by gel and analytical SEC (Figure 3.12), and the capacity and recovery of each can also be compared (Table 3.4). Qualitatively, the purity of both methods are comparable to NiNTA by gel. However, by analytical SEC protein purified by both test methods contained more high and low molecular weight contaminants. It is possible that these are non-protein contaminants since they are not observed in gel. Additionally, it is important to note that the protein had been sitting at 4C for four months longer than the sample purified by Nickel-IMAC. It is quite possible the protein aggregated during this time, particularly because GFP is present in this fusion protein. If that is not the case, it is possible that these methods could be further optimized to improve protein quality.

Table 3.4. Capacity by various immunoaffinity purification methods.

Purification Method	Recovery	Capacity of column
5mL Nickel-IMAC column using His-tag of IDL517	35mg: 20mL at 1.75mg/mL	7mg/mL
1mL ProteinY-CTpeptide mAb B40-NHS immunoaffinity column using CTpeptide-tag of IDL517	0.5mg: 2mL at 0.25mg/mL	0.5mg/mL
0.5mL cAbGFPmutant IDL652-NHS immunoaffinity column using GFP-tag of IDL517	1.65mg: 5mL at 0.33mg/mL	3.3mg/mL

Capacity of various immunoaffinity purification methods for proof of concept fusion protein IDL517.

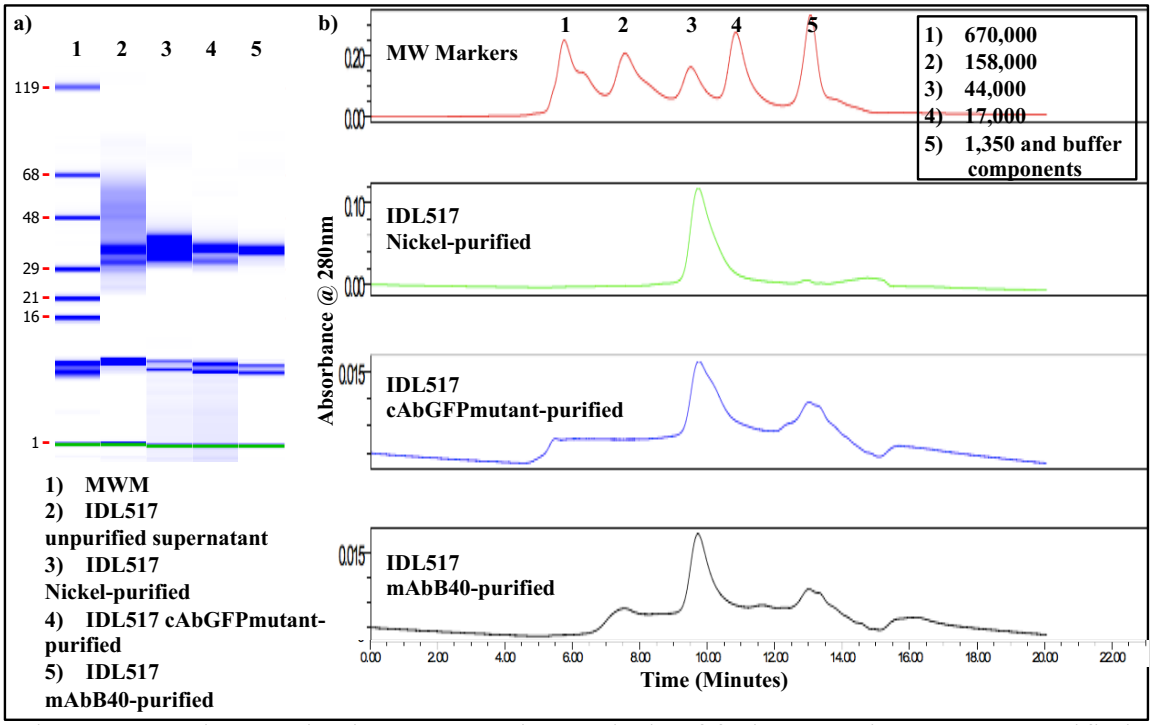


Figure 3.12. Characterization comparative analysis of fusion-protein **IDL1517** purified by various immunoaffinity purification methods (Nickel-, cAbGFPmutant(IDL652)-, and ProteinY-CTpeptide-mAbB40-purification), by **a** Gel-CHIP analysis (non-reduced) and **b** analytical SEC.

Chapter IV

Discussion

4.1. Overview

There are a variety of fusion-tags currently in use as tools for protein purification. Many of the current methods can be problematic for certain proteins, in particular, many purification strategies involve elution steps performed at low pH. This acidic pH can be harsh on pH-sensitive proteins, or on proteins containing a GFP-tag.

In this work, we applied a variety of in-house resources to design three novel immunoaffinity purification methods that could offer a gentler method for the purification of pH-sensitive proteins. The overall purpose of this study was to determine if the three test fusion-tags could be used in novel immunoaffinity purification schemes to successfully purify protein in gentler elution conditions, while achieving a purified result similar in quality to that of a well-established method, such as Nickel IMAC purification via His-tag.

The first affinity method, the anti-ProteinY-CTpeptide mAb affinity method, was hypothesized to act as an in-house, less costly, substitute for Flag™-tag, by fusing a small peptide sequence, derived from the N- or C-terminal sequence of Protein Y, to a recombinant protein. The peptide-fusion would be purified on an affinity column immobilized with an in-house antibody specific to the peptide sequence. Competitive elution of the peptide-fusion would be attempted with excess peptide in a neutral pH buffer.

The second method, the cAbGFPmutant affinity method, hypothesized that a

mutation or mutations could be introduced to the camelid antibody against GFP (cAbGFP4) amino acid sequence. The intended result of the mutation would be the alteration of the cAbGFP antibody's affinity for GFP at various pHs, yielding pH-dependent binding of GFP. Such binding conditions would thereby allow for an elution of GFP-fusion recombinant protein in a non-extreme pH buffer on a cAbGFPmutant-immobilized affinity column.

The third method, the mIgG1/Z domain affinity method, hypothesized that a synthetic Protein A domain, domain Z, could be fused (in ZZ form) to a recombinant protein and purified on a murine IgG1-immobilized column with a gentle elution at a neutral pH. The rationale is that affinity of Protein A for murine IgG1 is higher in high NaCl, high pH conditions, so decreasing the avidity effect, by using ZZ (double-domain) instead of Protein A (pentavalent), and decreasing the NaCl and pH during elution, would enhance this effect, allowing elution to occur when high NaCl and high pH were not present.

The data supported the first two fusion-tag hypotheses (ProteinY-peptide-fusion and GFP-fusion), and partially supported the third (ZZ-fusion). The anti-ProteinY-CTpeptide mAb affinity column was successfully used in a manner similar to a Flag™-tag, and was able to purify a CTpeptide-fusion protein, with binding occurring in PBS, and elution occurring with excess peptide at a neutral pH. The resulting protein had none of the contaminants or aggregates found in the unpurified supernatant.

To test the second hypothesis, a number of cAbGFPmutants were successfully designed and expressed, and Octet experiments indicated pH-sensitivity for a number of mutants. The pH-sensitive mutants may have the ability to bind GFP in non-extreme pH conditions, while maintaining a decreased binding ability to GFP at another non-extreme

pH, due to the loss of affinity for GFP at that pH. The GFP-fusion was successfully bound and eluted from the cAbGFPmutant affinity column, while also reducing contaminants and aggregates found in the unpurified supernatant.

Lastly, the murine IgG1 antibodies did have an affinity for ZZ-fusion, as well as Z-fusion, but in the case of the ZZ-tag, the affinity may have been too high to make use of it in the intended manner (a gentle PBS elution). And while the Z-tag seemed to have a higher recovery, based on what amount bound to and eluted from the column, the capacity itself was not high enough to make use of it as a robust purification method.

4.2. Anti-ProteinY-CTpeptide mAb affinity method

In the first method tested, a small peptide sequence, derived from the C-terminal of in-house Protein Y, was fused onto a recombinant protein. An antibody specific for the peptide was immobilized on an immunoaffinity column. The CTpeptide-fusion protein was purified on this immunoaffinity column, then gently eluted using excess synthetic CT-peptide in PBS.

Three anti-CTpeptide antibodies and three anti-NTpeptide antibodies were generated in a previous campaign by the Antibody Discovery Group at Biogen. These antibodies were first tested for binding to the peptides by ELISA. Binding data from ELISA (Figure 3.5) indicated that only the C-terminal peptide antibodies had affinity for their respective CT-peptide sequence, each displaying typical binding curves on C-terminal peptide-coated ELISA plate (Fig. 3.5b). No binding was observed with the three anti-NTpeptide to the C-terminal-peptide, as expected. On the NT-peptide-coated plate, no binding was observed for all three CTpeptide mAbs and for two of the NTpeptide mAbs (Fig. 3.5a). Anti-NTpeptide mAb A10 displayed weak binding on the NTpeptide

coated plate. The results with the anti-NTpeptide mAbs were surprising since these antibodies had been previously selected by our antibody discovery group for their specificity for the NTpeptide. Further investigation revealed that the ELISA conditions used by the Antibody Discovery Group included higher peptide coating density and higher antibody concentrations. These differences in ELISA conditions explain why these antibodies were originally selected as NTpeptide specific, yet no binding was observed in these studies. These studies confirmed that indeed these antibodies were not of sufficient affinity to be useful for immunoaffinity purification.

All six mAbs were tested in column format by binding to Protein A beads and then adding fusion protein IDL130, which contains both ProteinY-terminal peptide tags. Samples were taken at each step and analyzed by gel to determine where the fusion protein was located. In the mAb A10 sample (Figure 3.6a), there was fusion protein in the flow-through following binding to mAb A10-Protein A beads (Fig. 3.6a.2), and none in the bead sample following this load (Fig. 3.6a.3). Additionally, when eluted with excess peptide, there was no fusion protein in the flow-through (Fig 3.6a.4,5). The result of both of these steps indicate that the fusion protein never bound to the mAb column. The resin was tested after peptide elution, and only mAb A10 remained (Fig. 3.6a.6). It was then eluted with low pH buffer to further confirm that no fusion protein bound, but could not be eluted with peptide. The result of this was, again, only mAb A10 in the sample Fig. (3.6a.7). Based on this result, all three anti-NTerminal-peptide mAbs were dismissed as possible immunoaffinity antibodies.

In contrast, all three of the anti-CTerminal-peptide mAbs were successful in the same small-scale elution test. The Protein A bead test of the candidate (mAb B40) selected for further tests is shown (Fig. 3.6b). There was fusion protein present in the

initial flow-through following binding to the mAb-Protein A resin (Fig. 3.6b.2). This was likely an overload of fusion protein, and meant that there was excess fusion than the capacity of the column. In addition, the resin sample still contained fusion protein after loading (Fig. 3.6b.3), and fusion protein was still present in both peptide elution fractions (Fig. 3.6b.4,5). Following elution, the resin itself was tested, and very little, if any fusion protein remained bound to the mAb (Fig. 3.6b.6). When low pH buffer was used for follow-up elution, only mAb B40 was present in the flow-through (Fig. 3.6b.7), indicating that the fusion protein was completely eluted in the peptide elution fractions.

These results were then translated into a column format, in which anti-CTpeptide-mAb B40 was covalently immobilized to NHS-activated sepharose. This format is preferable for immunoaffinity purification because the likelihood of antibody leaching off of the column during the purification is minimal. In the test format with the antibody just bound to protein A sepharose and not covalently attached, antibody could potentially leach from the column, both reducing capacity and possibly contaminating the fusion protein. When used for the purification of CTpeptide-fusion IDL517, the recovery was 0.5mg of purified protein (Figure 3.7), or 0.5mg/mL of resin. In contrast, the Nickel-purification of this fusion protein resulted in a capacity of 7mg/mL of resin (Appendix 1.2). While the Nickel-purification method has a higher capacity than the ProteinY-CTpeptide mAb immunoaffinity purification method, the capacity is equivalent to that of Sigma's Anti-Flag® M2 Affinity Gel, whose resin-binding capacity is advertised as >0.6mg/mL (Sigma, A2220). So although it does not match the capacity of Nickel-IMAC, it has very comparable capacity to alternate peptide elution methods.

There are a number of reasons that could explain this low capacity. In this work, the antibodies were crosslinked to NHS-activated agarose. The chemical reaction

enabling the conjugation to occur is the reaction between the NHS-ester group (activated on the solid support resin) and the primary amines ($-NH_2$) of the antibody, resulting in the release of the NHS group and the formation of a stable amide bond between the protein and the ester (ThermoFisher, Amine Reactive Crosslinker Chemistry). Primary amine groups are found on lysines, as well as at the N-terminus of the protein, therefore the reaction occurs specifically, but randomly, making it highly probable that at least some of the antibody was conjugated in such an orientation to make it inaccessible, or less accessible, to the synthetic peptide-fusion protein. It is also possible that conjugating certain lysine residues could interfere with the antibody's binding site. So while NHS-activated agarose is a good tool for stable conjugation of antibody, it is not as efficient in terms of antibody placement and overall ability to bind antigen.

To improve capacity, there are other crosslinking methods exist that are less stable, such as crosslinking the antibody directly to Protein A beads using the reagent dimethyl pimelimidate (ThermoFisher, Chemistry of Crosslinking). This reaction is reversible at high pHs, however, so is not a good method for producing a solid, stable, immunoaffinity resin, but may be useful as an experimental resin. One other crosslinking method that should have been explored, and may be useful in the future, is the Pierce™ Protein A IgG Plus Orientation Kit (ThermoFisher, 44893). According to the manufacturer, it is more stable than the dimethyl pimelimidate method, resulting in less antibody-leaching. It also binds and crosslinks the IgG so that the antigen binding site of the antibody always faces outward. This would certainly help any capacity issues associated with suboptimal orientation of the antibody.

Finally, to compare characterization of the two methods, the gel indicates a more pure sample by ProteinY-CTpeptide immunoaffinity method than by Nickel-IMAC

method (Figure 3.8a). The analytical SEC indicates possible aggregate, as well as some low molecular weight contaminant (Figure 3.8b). It is likely that the low molecular weight contaminant is actually excess peptide remaining in the sample, since it was never treated post-affinity purification, and a high concentration of peptide was used for elution. This could be easily removed by dialysis. That aside, the peaks run on top of one another, indicating equivalent purity. A possible cause for the aggregate may be time. It is interesting to note that in both this sample, and the sample purified (discussed in 4.3) by cAbGFPmutant immunoaffinity method, the analytical SEC traces look quite similar. Both have a small peak of what appears to be aggregate. Both samples went through their final purification method testing four months later than the initial Nickel-purifications of the same sample. It is possible that the sample sitting at 4°C for four months is the cause of this aggregate, and it is unable to be removed by either method. Had these methods been tested much earlier, it is possible their results would be more similar in low-aggregate form to the Nickel-purified sample. In fact, it may have been prudent to re-purify a sample by Nickel alongside these to see if the aggregate also appeared in that purification method. The presence of GFP in this fusion protein suggests that aggregation may be the cause of the peak, since GFP tends to aggregate. It is also possible that the aggregate occurring at 4°C is specific to this particular fusion protein. It would be interesting to see if the aggregation occurs in a different fusion protein containing this synthetic peptide tag. If that is not the cause of the aggregate, in the future it may be useful to test some wash steps between the load and peptide elution steps used in this test. A more stringent wash could be used to remove any aggregate prior to elution, such as a citrate buffer at pH6.0, which due to its neutral pH should not elute any protein of interest.

Although this peptide-elution method has some drawbacks, particularly in the capacity area, it still has benefits to its use. In general, the low cost of this method, due to the in-house resources – particularly that the mAb is readily available in large quantities – may make it more useful than a Flag™-tag, which can become quite costly for large-scale purifications. Additionally, the method seems to be efficient. No protein was eluted in a follow-up step using a low pH2.8 elution buffer, indicating that the peptide elution had successfully eluted all of the bound protein. So although the capacity is not as high as other methods, it does recover what is loaded, which is important when dealing with low-supply or low-expressing proteins. It also results in a high-quality, low-aggregate, product, and compares to anti-Flag affinity purification. It may also be useful for the purification of samples from lysate, such as SF9-expressed protein. When the His-tag is used to purify cell lysates, the result is never as clean due to non-specific binding. Lastly, this could be used similarly to how Flag™ is often used, for purification of low-expressing lysates. Due to time constraints, this application could not be tested, but it would be interesting to test in the future.

4.3. cAbGFPmutant affinity method

In the second purification method investigated, a GFP tag was added to a recombinant protein and the fusion protein was purified on an immunoaffinity column using an engineered anti-GFP antibody. The anti-GFP camelid derived antibody was engineered to possess pH-dependent binding to GFP. The GFP-fusion protein was bound to the column at pH 5.5 and eluted at pH 9.5.

Numerous cAbGFPmutants were screened against GFP to determine a mutant form that bound GFP at pH5.5, but lost its affinity for GFP around pH9.5. Binding data

from Octet Kinetic Analysis suggested approximately eight mutants that may have such qualities. The results of Octet (Table 3.2) indicated that mutants IDL202, IDL206, IDL649, IDL650, IDL652, and IDL656 did not bind GFP at pH9.5. In addition, IDL649, IDL650, and IDL652 did bind GFP at pH5.5. IDL653 and IDL655 also had pH-sensitivity, but low binding overall. After Octet narrowed the mutants down to eight candidates, each was tested in a small-scale on-resin purification (Figure 3.9a). In the first test, IDL650, IDL652, and IDL653 had GFP in the pH9.5 elutions, in agreement with the Octet results. These three mutants were further tested using different conditions to determine the optimal mutant (Fig. 3.9b,c). When using less cAbGFPmutant and eluting at pH9.5, IDL652 had the best elution results of the three, where green was visually observed in its elution fractions, and very little green remained on the resin itself. IDL652 was tested alone, using various cAbGFPmutant concentrations. Green was observed in the middle of the elution fractions, and the first and last fractions were clear (data not shown). This indicated that the pH9.5 elution buffer was a sufficient buffer for elution of protein, able to elute the majority of bound GFP.

The results were then translated to an on-column format, in which cAbGFPmutant IDL652 was covalently immobilized to NHS-activated sepharose. As mentioned in the CTpeptide-fusion discussion, this format is preferable for immunoaffinity purification because the likelihood of antibody leaching off of the column during the purification is minimal (see 4.2). When used for the purification of GFP-fusion IDL517, the recovery was 1.65mg of purified protein (Figure 3.10), or 3.3mg/mL of resin. In contrast, the Nickel-purification of this fusion protein resulted in a capacity of 7mg/mL of resin (Appendix 1.2). While the Nickel-purification method has a higher capacity than the anti-GFP immunoaffinity purification method, the capacity is still acceptable.

There are similar reasons to the anti-peptide purification method for why there may be a lower than expected capacity. The conjugation specific for lysine, but randomly occurring on the antibody, makes it highly probable that at least some of the antibody was conjugated in such an orientation to make it inaccessible, or less accessible, to the synthetic peptide-fusion protein. It is also possible that conjugating certain lysine residues could interfere with the antibody's binding site. So while NHS-activated agarose is a good tool for stable conjugation of antibody, it is not as efficient in terms of antibody placement and overall ability to bind antigen.

To improve the capacity, the same conjugation methods could be employed as described in the peptide-fusion purification section above (4.2), namely attempting to conjugate antibody to Protein A resin using crosslinking reagent dimethyl pimelimidate, or using the Pirece Protein A IgG Plus Orientation Kit (ThermoFisher, 44893) to improve antibody orientation.

In terms of quality, comparison of characterization between this method and the Nickel-purification method indicates similar levels of purity by gel (Figure 3.11a). Like with the peptide elution method, the analytical SEC (Figure 3.11b) indicates possible aggregate, as well as some low molecular weight contaminant. A possible cause for the aggregate may be time. It is interesting to note that in both this sample, and the sample purified by peptide mAb immunoaffinity method, the analytical SEC traces look quite similar. Both have a small high-molecular weight peak of what appears to be aggregate. Both samples went through their final purification method testing four months later than the initial Nickel-purifications of the same sample. It is possible that the sample sitting at 4°C for four months is the cause of this aggregate, and it is unable to be removed by either method. Had these methods been tested much earlier, it is possible their results

would be more similar in low-aggregate form to the Nickel-purified sample. In fact, it may have been prudent to re-purify a sample by Nickel alongside these. If the GFP is not the cause of the aggregate, it may be useful in the future to test some wash steps between the load and peptide elution steps used in this test. A more stringent wash could be used to remove any aggregate prior to elution, such as a citrate buffer at pH6.0, which due to its neutral pH should not elute any protein of interest.

Although this cAbGFPmutant method does not have quite the capacity that Nickel-purification does, the capacity is not low enough to rule it out as an effective purification method. It may also be more useful to purify via GFP-tag than his-tag when dealing with lysates, as a His will not clean up a lysate the way a more specific cAbGFPmutant would. Additionally, it can be used to purify GFP-fusions without negatively affecting the GFP, or the protein, since it can be efficiently eluted without a harsh extreme pH buffer (such as pH2.8). Like the peptide mAb immunoaffinity method, the cAbGFPmutant is produced in-house, which greatly decreases the cost associated with immunoaffinity resins, making it a very attractive option for large-scale purifications. Lastly, although the capacity is not as high as a Nickel-IMAC resin, it is still high enough to be a useful purification tool, and the purification efficiency is high, not only allowing for purification of a protein, but for high recovery of the protein, which is important when dealing with low-expressing proteins.

4.4. Murine IgG1 affinity method for ZZ-fusion purification

In the last method tested, a ZZ domain was fused to a recombinant protein and the fusion protein was purified on a murine IgG1 column. The ZZ domain is derived from Protein A and is known to bind the Fc portion of antibodies. Because murine IgG1 binds

to Protein A only in high pH and high NaCl conditions, binding of the fusion protein to the murine IgG1 column was performed in these conditions and the protein was eluted using PBS (reduced pH and NaCl conditions).

A number of antibodies were screened against the ZZ-fusion, as well as against a Z-fusion, in case the ZZ-domain proved to have too high an avidity for the murine IgG1. Octet analysis (data not shown) was difficult to interpret. It indicated that even the negative control, a murine IgG2a which has a high affinity for Protein A in PBS conditions, and therefore will not easily separate from Protein A at such conditions, easily dissociated from ZZ-fusion and Z-fusion proteins in the PBS dissociation step. This occurred no matter what tip was used – Protein A, Protein G, or anti-murine capture tips – and indicated that Octet results could not be easily translated to a column format.

Since Octet did not prove to be a useful screening tool, all of the murine IgG1 antibodies, as well as a control murine IgG2a control, were covalently immobilized to NHS-activated sepharose, so that the method could be tested in an on-column format. When used for purification of the ZZ-fusion IDL130, approximately 2mg of fusion was loaded onto each murine IgG1 affinity column. Based on the absorbance of the flow-throughs, washes, and elutions, between 1.3—1.8mg bound to the column in each murine IgG1. However, no more than 0.4mg eluted. Of the protein that bound, only 15%, 15%, and 23% of ZZ-fusion IDL130 was eluted with PBS from the murine IgG1-immobilized affinity columns (Table 3.3).

In contrast, the three mIgG1 and the mIgG2a column were also tested for purification of Z-fusion IDL519. Approximately 2.4mg of Z-fusion protein was loaded onto each of the four columns. In this case, very little actually bound, at most less than 0.6mg, by the anti-R murine IgG1. The other two murine IgG1s each bound less than

0.25mg. The murine IgG2a, however, bound 1.7mg, and as a positive control for binding, this makes sense. Of the little Z-fusion protein that was bound by the three murine IgG1-columns, less than 0.2mg was eluted from each. For the mIgG2a control, 0.04mg was eluted, for a 2% recovery rate of bound material. Although Anti-H WT and Anti-H agly were able to elute 82% and 60% of the bound material, respectively, the capacity each had was much too low to make use as a robust purification method. Only 0.08mg bound to anti-H WT, for a capacity of 0.08mg/mL resin. For anti-H agly, the capacity was 0.25mg/mL of resin (Table 3.3). This is half as much as the capacity of ProteinY-CTpeptide mAb resin or anti-Flag resin, and each of those have a better recovery. ProteinY-CTpeptide recovered 3-4x as much as either of these murine IgG1, and less protein was loaded for purification. However, these cannot be directly compared since they were used to purify a different fusion protein (IDL517) than the mIgG1/ZZ/Z method. But in contrast to this, the Nickel-purification of the same Z-fusion protein IDL519 resulted in a capacity of 5mg/mL of resin, and the Nickel-purification of the ZZ-fusion protein IDL130 resulted in a capacity of 6mg/mL of resin. So the Nickel-purification method has a much higher capacity than the murine IgG1 immunoaffinity purification method, and results in a relatively pure, aggregate-free sample.

While the proposed method works fine in an opposite manner, in which murine IgG1 antibody is purified by Protein A column in the described conditions, the converse method did not translate. So although in theory the ZZ-fusion/mIgG1 purification method should work, it is possible that an avidity effect is at play here, making it difficult to fully elute the protein from the murine IgG1 immunoaffinity column. This seems quite likely, since more ZZ-fusion protein bound to the column than Z-fusion protein, even when more of the Z-fusion was loaded, indicating the affinity between ZZ and murine IgG1 is

higher than that between Z and murine IgG1. At the same time, although more protein bound to the columns in the case of the ZZ-fusion, (between 3.2 and 16.5x more than Z-fusion), this did not translate to more eluting. Only about twice the amount of ZZ-fusion eluted from a column than Z-fusion. It is possible in the case of ZZ that the material came off in wash steps and was not accounted for, in which case the capacity issue is still in play.

Another reason for the low capacity of the murine IgG1-affinity columns could be one that was mentioned for the previous two purification methods. The coupling of antibody to NHS-activated agarose by way of lysine residues could again have a negative effect on the binding ability of the antibody, as well as decreasing the availability of the Fc due to its binding orientation. If the capacity issue could be rectified, using the suggested methods already described in 4.2 and 4.3, it is still unlikely that this method would be useful, because the avidity effect of the ZZ-fusion would still be a problem, and the fusion protein would still be unable to be eluted from the column.

For this work, the ZZ-fusion/Z-fusion purification by murine IgG1-NHS resin was not as successful as hypothesized. The ZZ-fusion bound to the immunoaffinity column, but did not elute well, which are likely the result of an avidity effect. In contrast, the Z-fusion did not bind well, but what did bind was relatively efficiently eluted with PBS, despite the low binding capacity. So while this method was unsuccessful in purifying either of the two fusion proteins, it cannot be completely ignored as a possible purification method. It has some upside, particularly in making use of the Z-fusion. It is possible that the method could be tweaked prior to using it for a preparative purification, as a way to either somehow increase the binding capacity for the Z-fusion, without greatly increasing the affinity for it in the load step. Perhaps engineering experiments

similar to the cAbGFPmutant work could be performed, in which some mutations were introduced to the Z-fusion that may alter the binding between it and murine IgG1, but in this case, the mutations would be made as a way to improve binding during the high pH load step, instead of to decrease binding at a high pH elution step. On the other hand, this may be too much work for too little gain, in which case this method can be dismissed as a useful purification tool.

4.5. Conclusion

The three purification methods, a cAbGFPmutant immunoaffinity resin to purify a GFP-fusion, an anti-ProteinY-CTpeptide mAb immunoaffinity resin to purify a ProteinY-CTpeptide-fusion, and a murine IgG1 immunoaffinity resin to purify a ZZ-fusion protein, all had elements of success. The cAbGFPmutant purification method and the anti-ProteinCTpeptide mAb purification method were the most successful, resulting in protein as pure or purer than that resulting from a His-tagged Nickel IMAC purification method. The murine IgG1-immobilized resin method was slightly successful, however, the capacity issue is too big to ignore, and it is unlikely to be truly useful for large-scale purification.

The cAbGFPmutant affinity purification method had the greatest capacity of the three test methods. Combined with the in-house availability of the antibody, this method could serve well in-house, and is certainly a good alternative to previously-established GFP purification methods. The ability to be eluted at a neutral pH is an attractive quality of a fusion-tag.

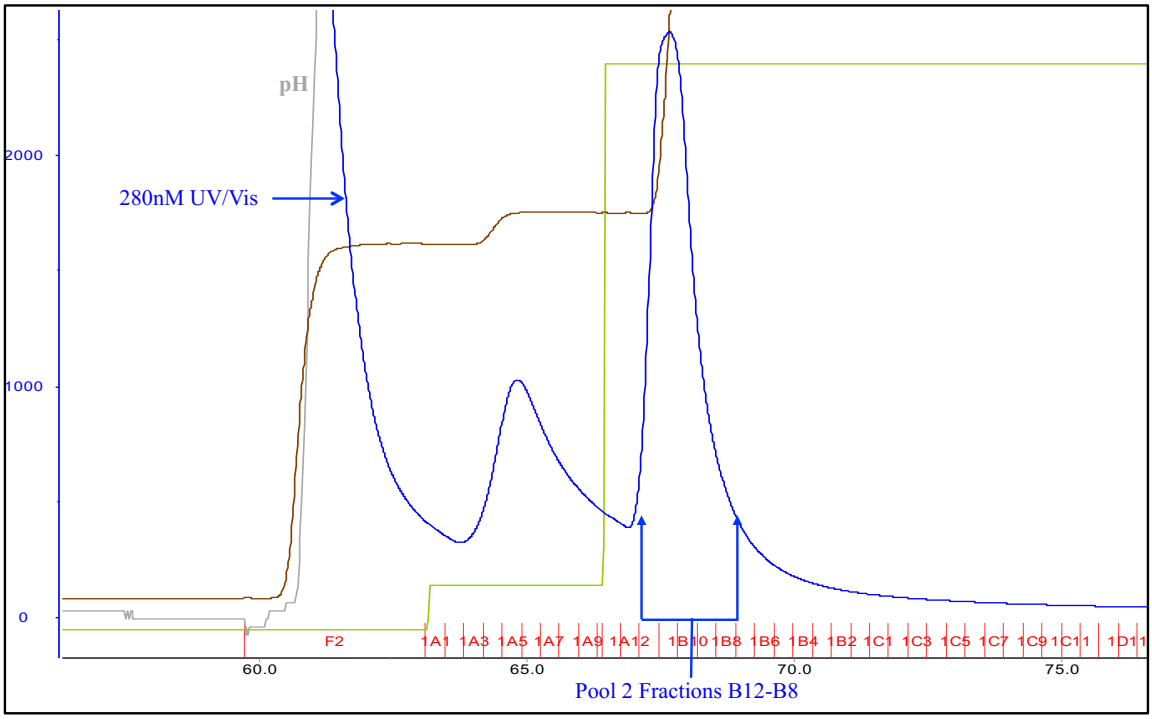
The ProteinY-peptide mAb affinity purification method, although low capacity, is equivalent to purification of a Flag™-tag-fusion by anti-Flag™ resin. In addition, the

great cost savings assumed by the in-house production of anti-ProteinY-CTpeptide mAb makes it a very attractive fusion-tag for use in-house. The small size of the tag also makes it a great option for difficult to purify, or large proteins. It also makes it an easy recombinant protein to express, by not adding much metabolic burden to the cells.

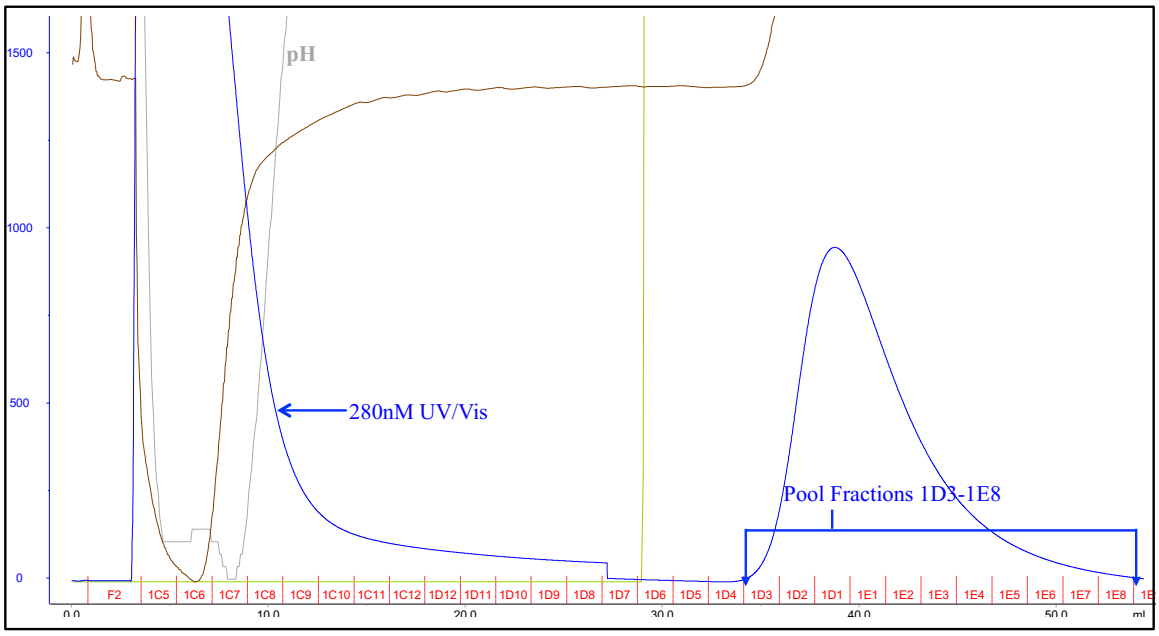
It is possible to tweak these two methods even more to further improve upon their usability. The fusion protein constructs used in this study consisted of a number of fusion tags. It would be interesting to see how much more beneficial they could be on a protein consisting of only the one fusion-tag. This may help with capacity and purity issues. It would also be interesting to use a variety of conjugation methods, such as the two described earlier, to stably bind the antibody to a resin matrix. This could also help with capacity issues. Combining these suggestions could have great consequences for the capacity of these proposed immunoaffinity purification methods.

Appendix

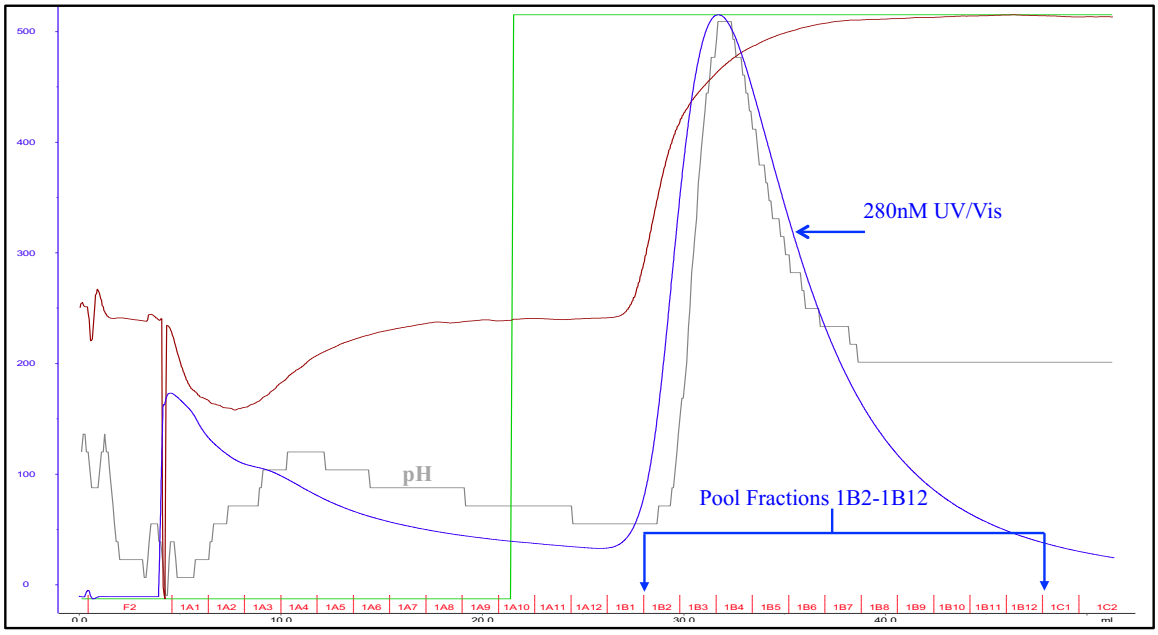
Appendix 1. Chromatagrams of Nickel-purification of the three proof-of-concept fusion proteins.



Appendix 1.1. Chromatogram of Nickel-IMAC purification (on 5ml column) of Fusion Protein **IDL130** supernatant. Peak 1 (fractions A3-A9) was less pure and contained more aggregate than Peak 2 (fractions B12-B8). Peak 2 was collected and contained 30mg of purified material. Its characterization can be seen in Figure 3.2.



Appendix 1.2. Chromatogram of Nickel-IMAC purification (on 5ml column) of Fusion Protein **IDL517** supernatant. Pool contained 35mg of material. Its characterization can be seen in Figure 3.3.

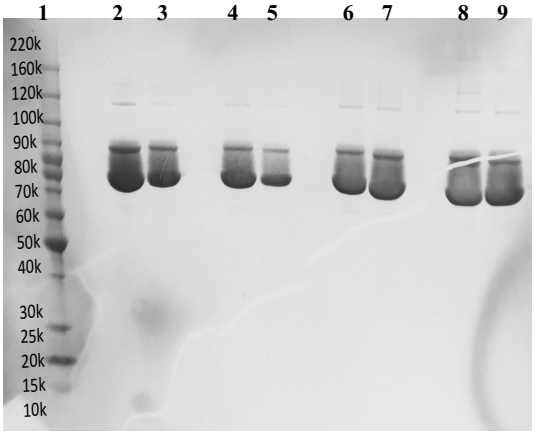


Appendix 1.3. Chromatogram of Nickel-IMAC purification (on 5ml column) of Fusion Protein **IDL519** supernatant. Pool contained 25mg of material. Its characterization can be seen in Figure 3.4.

Appendix 2. Mutations, Octet Kinetics, Theoretical MW, and Theoretical pI of 36 cAbGFPmutant constructs.

Construct ID	Number of mutations to Histidine	Octet Kinetic Analysis of GFP-binding at pH5.5, pH9.0 and/or pH9.5	Theoretical MW (kDa)	Theoretical pI
IDL031	0 (Wild-type)	Binding less efficient than pH9.0	38186.9	7.16
IDL032	1H	Binding less efficient than pH9.0	38137.9	7.19
IDL033	1H	Binding less efficient than pH9.0	38176.9	7.19
IDL034	2H	Binding less efficient than pH9.0	38127.8	7.22
IDL191	1H	Binding less efficient than pH9.0	38160.9	7.19
IDL192	1H	Binding less efficient than pH9.0	38237	7.19
IDL193	1H	Good binding at 5.5, slight pH sensitivity	38167.9	6.78
IDL194	1H	Binding less efficient than pH9.0	38160.9	7.19
IDL195	1H	Binding less efficient than pH9.0	38128.9	7.68
IDL196	1H	Binding less efficient than pH9.0	38237	7.19
IDL197	1H	Binding less efficient than pH9.0	38253	7.19
IDL198	1H	Binding less efficient than pH9.0	38209	7.69
IDL199	1H	Binding less efficient than pH9.0	38167.9	6.78
IDL200	1H	Binding less efficient than pH9.0	38237	7.19
IDL201	1H	Binding less efficient than pH9.0	38210	7.19
IDL202	1H	No binding at pH9.5	38195	7.69
IDL203	3H	Binding less efficient than pH9.0	38159.9	7.22
IDL204	3H	Binding less efficient than pH9.0	38131.9	7.7
IDL205	3H	Binding less efficient than pH9.0	38245	7.7
IDL206	2H	No binding at pH9.5	38136.9	8.07
IDL240	cAbGFP4-hIgG1 (GS)	Binding less efficient than pH9.0	38186.9	7.16
IDL241	1H	Good binding at pH5.5, slight pH sensitivity at pH9.5	38167.9	6.78
IDL242	2H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38148.8	6.55
IDL243	2H	Binding less efficient than pH9.0	38157.9	6.82
IDL244	2H	Binding less efficient than pH9.0	38118.8	6.82
IDL245	2H	Very low binding overall	38218	6.82
IDL647	2H	Very low binding overall	38218	6.82
IDL648	2H	No binding at pH9.5	38141.9	6.82
IDL649	2H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38218	6.82
IDL650	3H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38198.9	6.59
IDL651	3H	No binding at pH9.5	38122.8	6.59
IDL652	3H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38138.8	6.59
IDL653	4H	Low binding overall, pH sensitive	38089.7	6.64
IDL654	3H	No binding at pH9.5	38131.8	6.86
IDL655	3H	Low binding overall, pH sensitive	38089.7	6.64
IDL656	3H	Good binding at pH5.5, significant pH sensitivity at pH9.5	38159	6.82

Appendix 3. Characterization by SDS-Page of eight cAbGFPmutant antibodies.



1) **MWM**
2) **IDL202**
3) **IDL206**
4) **IDL649**
5) **IDL650**
6) **IDL652**
7) **IDL653**
8) **IDL655**
9) **IDL656**

Appendix 3. SDS-Page of Protein A purification (on 5ml column) of 8 cAbGFPmutant antibody candidates. Each pool contained 12-35mg of material. IDL652 was selected as candidate for testing the proposed purification method.

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